The role of bacterial infection and inflammation in the generation of overactive bladder symptoms

Submitted by

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Abstract

There is substantial evidence of considerable insensitivity affecting the current tests used to screen for urinary infection. The studies within this thesis provide original work in examining the performance of recommended diagnostic tests for urinary tract infection, and explore the bacterial ecology of urinary infection and its associated urothelial inflammatory response in patients with symptoms of overactive bladder.

The association between lower urinary tract inflammation, bacterial colonisation and the generation of overactive bladder symptoms was explored. An enhanced bacterial culture method was used and quantitative thresholds discredited. Comparative data from patients and controls demonstrated that bacterial urinary infection was evident in ninety percent of patients. Quantitative and qualitative differences in the bacterial ecology were found amongst patients and controls. These were associated with increased urothelial inflammation amongst patients.

Adenosine-5'-triphosphate (ATP) is a neurotransmitter and inflammatory cytokine implicated in the pathophysiology of lower urinary tract disease. ATP additionally reflects microbial biomass, thus may function as a surrogate marker of urinary tract infection (UTI). The potential of urinary ATP in the assessment of lower urinary tract symptoms, infection and inflammation was tested. Sampling techniques suitable for clinical practice were validated. Urinary ATP was reviewed as a marker of infection in patients with lower urinary tract symptoms. Though it may have a role as a research tool, it was unconvincing as a surrogate, clinical diagnostic marker. Several urinary cytokines were explored and urinary IL6 and Lactoferrin varied in relation to surrogate markers of infection in patients with overactive bladder symptoms.

Declaration

I, Kiren Gill, certify that the work presented in this thesis is the result of my own investigations, except where otherwise stated. Where information has been derived from other sources, this has been indicated. Professor Malone-Lee, Anthony Kupelian, Sanchutha Sathiananthamoorthy, Harry Horsley, Linda Collins and Sheela Swamy (Department of Medicine, Archway Campus, UCL, Highgate Hill, London N19 5LW) helped to collect urine specimens from patients and control volunteers.

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Dedication

I dedicate this work to my parents, Sukh and Esha.

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List of abbreviations

ABU	Asymptomatic bacteriuria
ACh	Acetylcholine
AE	Adverse event
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
AUM	Apical asymmetric unit membrane
BMI	Body mass index
BTX-A	Onabotulinum toxin A
cAMP	Cyclic adenosine monophosphate
CFU	Colony forming units
CI	Chief investigator
CNS	Central nervous system
CO_2	Carbon dioxide
CoNS	Coagulase-negative staphylococci
CRF	Case report form
CXCL 8	Interleukin 8
ELISA	Enzyme-linked immunosorbent assay
FISH	Fluorescence in-situ hybridisation
GCP	Good Clinical Practice
IBC	Intracellular bacterial community
ICS	International Continence Society
ICIQ – FLUTS	International consultation of incontinence modular questionnaire - Female Lower Urinary Tract Symptoms

IL6	Interleukin-6
IQR	Interquartile range
LPS	Lipopolysaccharide
LUTS	Lower urinary tract symptoms
MCP-1	Monocyte chemo-attractant protein 1
MHC	Major histocompatability complex
MCID	Minimal clinically important difference
ML	Millilitres
MSU	Midstream urine
NANC	Nonadrenergic, noncholinergic
NAT	Nucleic acid-based techniques
NF-κB	Nuclear factor kappa beta
NGF	Nerve growth factor
NO	Nitric oxide
NRES	National research Ethics Service
OAB	Overactive bladder
PRR	Pattern recognition receptor
PAG	Periaqueductal grey
PG	Prostaglandin
PMC	Pontine Micturition Centre
PVR	Post-void residual bladder volume
QIR	Quiescent intracellular reservoir
QoL	Quality of life
RCF	Relative centrifugal force
RCT	Randomised, controlled trial
RNA	Ribonucleic acid

RPM	Revolutions per minute
SAE	Serious adverse event
SAR	Serious adverse reaction
SOP	Standard operating procedure
SUI	Stress urinary incontinence
THP	Tamm Horsfall Protein
TLR4	Toll-like receptor 4
TCA	Tricyclic antidepressant
TCR	T cell receptor
UDS	Urodynamic studies
UK	United Kingdom
UTI	Urinary tract infection
UUI	Urgency urinary incontinence
UPEC	Uropathogenic E. coli
UP	Uroplakin
VBNC	Viable but nonculturable

List of publications as a result of this thesis

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1 Chapter 1 – Introduction

1.1 Epidemiology of lower urinary tract symptoms

Lower urinary tract symptoms (LUTS) is a collective term describing; 1) urinary storage problems such as frequency, urgency and urge incontinence; 2) voiding difficulties such as hesitancy, reduced stream, intermittency and incomplete voiding; 3) sensory symptoms that include experiences of pain; and 4) stress urinary incontinence (1, 2). Large population based studies have shown that LUTS is a global problem, affecting men and women, and increases with age. The EpiLUTS study collected data from 30,000 volunteers from US, UK and Sweden and reported the prevalence of at least one LUTS, at least 'sometimes', as 72.3% for men and 76.3% for women, and at least 'often' for 47.9% of men and 52.5% of women. The study reported that for most LUTS, at least half of the participants were bothered 'somewhat' or more and hence such symptoms have a significant impact on quality of life (3). Irwin et al estimated that in 2008, 45.2% of the world population was affected by at least one LUTS with an estimated rise to 2.3 billon (18.4% increase) by 2018 (4). In addition there is considerable overlap between these symptoms (3, 5) so that diagnostic categorisation may be difficult. These conditions are stigmatising (6) and are associated with a substantial economic burden (7, 8) with an estimated annual cost of £80 million to the NHS on continence products alone.

In women, risk factors for LUTS include the menopause, pregnancy and parity, mode of delivery and obesity. Women are more likely than men to have stress incontinence. Pregnancy, childbirth, menopause and normal female anatomy may account for this difference. However, men with prostate gland problems are at increased risk of urge

and overflow incontinence. Other risk factors include age, obesity, smoking and chronic cough, constipation and other medical conditions that may affect kidney function.

In patients presenting with LUTS, whatever the symptom mix, the exclusion of UTI is a mandatory first step in the assessment of all LUTS (9). Whilst acute UTI is not diagnostically challenging (10), in the case of LUTS without acute frequency and dysuria, exclusion of infection poses a diagnostic challenge.

1.2 Urinary tract system – Anatomy and function

The lower urinary tract contains the bladder and urethra. The micturition cycle describes the filling, storage and voluntary emptying of the bladder. The bladder is a muscular organ, which is situated below the peritoneal cavity near the pelvic floor. In men, it lies in front of the rectum and in women it lies in front of the uterus. Urine collects in the bladder from the kidneys via both ureters, which open into the bladder though ureteric orifices. Urine is expelled from the bladder through the urethra, a single muscular tube, which ends in the urethral orifice. Anatomically, the bladder is divided into a dome, two ureteric orifices, and an opening for the urethra, which surrounds the trigone at the base of the bladder. In men, the prostate gland lies outside the opening for the urethra (11).

The bladder is composed of three layers consisting of the mucosal layer, the smooth muscle known as the detrusor and the serosal surface. The mucosa has been described to consist of the urothelium, which is exposed to urine, a single layer of cells known the basement membrane and the lamina propria, which is an interface between the

mucosa and bladder muscle (12, 13). The muscular layer of the bladder is known as the detrusor, which consists of smooth muscle, with bundles know as fascicles.

The innervation of the body of the bladder differs from the bladder outlet, where the body is rich in beta-adrenergic receptors. These receptors are stimulated by the sympathetic component of the autonomic nervous system (ANS). Beta stimulation, via fibres of the hypogastric nerve, suppresses contraction of the detrusor. Conversely, parasympathetic stimulation, by fibres from the pelvic nerve and plexus, causes the detrusor to contract. Sympathetic stimulation is thought to be predominant during bladder filling, and the parasympathetic thought to cause emptying (14). The urethra, though anatomically differ in males and females, functions similarly. The bladder outlet is composed of the urethral sphincter consisting of the internal and external sphincter. The internal sphincter is composed of smooth muscle like the detrusor and extends into the bladder neck. Like the detrusor, the internal sphincter is controlled by the ANS and is normally closed. The primary receptors in the bladder neck are alpha-adrenergic. Sympathetic stimulation of these alpha-receptors, via fibres in the hypogastric nerve, is thought to contribute to urinary continence. The external sphincter is histologically different from the detrusor and internal sphincter, and consists of striated muscle under voluntary control. It receives its innervation from the pudendal nerve, arising from the ventral horns of the sacral cord (15) (16). During micturition, supraspinal centres block stimulation by the hypogastric and pudendal nerves. This relaxes the internal and external sphincters and removes the sympathetic inhibition of the parasympathetic receptors. This results in the voluntary passage of urine when the detrusor contracts allowing voiding of urine (17) (18) .

1.3 Overactive bladder

1.3.1 Definition

The definition of overactive bladder (OAB) has changed over time and is now expressed as a condition where there is urgency, the sudden overwhelming desire to pass urine (1). It may present with or without urge incontinence and a frequency of greater than 8 voids per day and nocturia of more than 1 (19). The prevalence of OAB is thought to be 16% and is commonly associated with other LUTS. It poses a substantial social-economic burden, which is ever growing with the aging population (8). It has a negative impact on quality of life affecting social, psychological, occupational, physical, and sexual aspects of those who suffer from it.

1.3.2 Symptom complex and assessment

The lower urinary tract symptoms expressed by patients with overactive bladder overlap with other lower urinary tract disease such as interstitial cystitis, painful bladder syndrome and acute urinary tract infection. The National Institute of Clinical Excellence (NICE 2015) states that the clinical history should be sufficient to diagnose and differentiate between lower urinary tract syndromes. There are many validated urgency questionnaires in the literature that help to diagnose OAB (20-24). Other validated questionnaires commonly used to assess lower urinary tract symptoms include the ICIQ–FLUTS, ICIQ-MLUTS and pain scores.

1.3.3 Anatomy and physiology of normal bladder and the OAB bladder

The urinary bladder is a musculomembranous sac, which acts as a reservoir for urine and lies in the anterior pelvis, behind the pubic symphysis, in both sexes. The urine

enters via the ureters and is expelled via the urethra. Superiorly the bladder is covered by peritoneum and lies anterior to the rectum, and uterus in females. The bladder wall, also known as the detrusor muscle, is made up of smooth muscle fibres arranged in spiral, longitudinal and circular bundles. The base of the bladder, known as the trigone, is bound laterally by the urethral orifices, with the internal urethral meatus anteriorly. The bladder is lined by a layer of transitional epithelium known as the urothelium.

The male and female urethra show marked anatomical differences, though function in a similar way. The male urethra is longer, approximately 20cm, encased by the prostate gland at the base of the bladder and the urethral sphincter. The female urethra is shorter, only 3-4cm long and descend through the pelvic floor. The urethra has both smooth and striated muscle types and continence is achieved by voluntary contraction of the external urethral sphincter. The normal bladder functions through a complex coordination of musculoskeletal, neurologic, and psychological functions that allow filling and emptying of the bladder contents. Continence is achieved by the synergistic relaxation of detrusor muscles and contraction of the external urethral sphincter.

1.3.3.1 Neurological control of micturition

Parasympathetic fibres, arising as preganglionic axons from S2 to S4, relay through ganglia mostly within the detrusor muscle. Postganglionic cholingeric nerves supply the detrusor muscle and stimulate detrusor contractions. Sympathetic nerves arise from T10 to L2 and relay in the pelvic ganglia. Their exact role in the control of micturition is unclear. It is known that α-adrenergic receptors and their nerve terminals are found mainly in the smooth muscle of the bladder neck and proximal urethra, where as β-receptors are found in the fundus of the bladder. The α-receptors respond to noradrenalin by stimulating contraction, where as the β-receptors relax the smooth

muscle. It is possible that the sympathetic neurons play a role in both urethral closure and detrusor relaxation during the filling phase of the micturition cycle.

The distal sphincter mechanism is innervated from the sacral segments S2-S4 by somatic motor fibres that reach the sphincter either by the pelvic plexus or via the pudendal nerves. Afferent nerves are carried in both the parasympathetic and pudendal pathways and transmit sensory impulses from the bladder, urethra and pelvic floor. These sensory impulses pass to the cerebral cortex and the micturition centre, where they produce reflex bladder relaxation and increased tone in the distal sphincter to maintain continence. Cortical control is a basic part of the micturition cycle. The higher centres suppress detrusor contractions and their main function is to inhibit micturition. Afferent impulses pass to the brain via the posterior columns to the pons, periaqueducatal grey, the anterior cingulate gyrus and the preioptic area of the hypothalamus.

1.3.3.2 The Micturition Cycle

The bladder alternates between a storage/filling phase and an emptying/micturition phase. During the storage phase the bladder is able to fill steadily without rise in the intravesical pressure due to the high compliance of the detrusor muscle. As the volume of urine increases, stretch receptors in the bladder are stimulated resulting in reflex bladder relaxation and reflex increase in sphincter tone. At 75% capacity there is the first desire to void but voluntary control is exerted over the desire, which then temporarily disappears. Compliance of the detrusor muscle allows further filling until the next desire to void and the emptying phase. Micturition is coordinated by the pontine micturition centre (PMC) in the pons. It is initiated first by voluntary and then by reflex relaxation of the pelvic floor and distal sphincter mechanisms, followed by

reflex detrusor contraction. Intravesical pressure remains greater than urethral pressure until the bladder is empty. The normal control of micturition requires coordinated reflex activity of the autonomic and somatic nerves. These responses depend on normal anatomical structures and normal innervation. Hence structural or neurogenic lesions can lead to disorders of micturition.

Symptoms of overactive bladder were previously thought to be synonymous with urodynamic detrusor overactivity. Hashim et al found that only 64% of patients with symptoms of overactive bladder demonstrated urodynamic detrusor overactive (DO) with more than 30% of patients with no OAB showing DO on filling cystometry (25). It has more recently been proposed that OAB is a symptom complex and several theoretical propitiations are published regarding the underlying pathology.

1.3.3.3 OAB – Myogenic proposition

The myogenic proposition states that changes in the detrusor muscle are responsible for involuntary detrusor contractions. It is thought that a local contraction is able to spread and cause a co-ordinated contraction of the bladder wall. Detrusor overactivity has also been associated with changes in the muscle architecture including increased gap-junctions, which could influence spread of muscle contractions. These changes have also been observed in the bladder as part of aging and also in conditions such as bladder outlet obstruction leading to detrusor hypertrophy (26).

1.3.3.4 OAB – Neurogenic proposition

It has been suggested that damage to the central pathways or sensitisation of peripheral afferent nerve may induce voiding reflexes which trigger detrusor overactivity (27). Mechanism that have been proposed in the generation of DO include

insult to the brain or spinal cord resulting in reduction in the suprapontine suppression, expression of primitive spinal bladder reflexes, synaptic plasticity, and sensitisation of peripheral afferent terminals in the bladder.

1.3.3.5 OAB – Autonomous proposition

The autonomous proposition proposes that the detrusor is modular, with each module being supplied by an individual bladder ganglia or by a node of interstitial cells known as a myovescial plexus. During normal filling there is autonomous activity with non-micturition contractions and phasic sensory discharge. In pathological conditions there is thought to be augmentation of autonomous activity resulting in detrusor overactivity. In addition increased communication between modules is also thought to result in increased detrusor activity. In summary the pathophysiology of DO included damage to the afferent and efferent pathways to and from the bladder. The true aetiology of OAB/DO may vary in different individuals and co-exist, as well as including other mechanisms, which have not yet been found.

1.3.3.6 The role of the urothelium in OAB

The urothelium is no longer thought to be an inert structure but is recognised to have an important sensory role in bladder function. The urothelium is thought to exhibit neuron like properties with expression of messenger molecule receptors and is the site of action of various neurotransmitters that play a role in the peripheral control mechanism of bladder activity (28). In addition, it has been suggested that there is direct communication with sub-urothelial afferents. Ferguson et al showed that when mouse detrusor muscle is stretched, flux of calcium intracellularly results in the release of ATP on the serosal aspect of the urothelium (29). It is now thought that ATP is released by both surfaces of the urothelium (30). ATP activation of purinergic receptors, P2X3 and/or P2X2/3, on suburothelial afferent nerves leads to relay of information to

the central nervous system to produce a sensation of bladder fullness and urgency (31). Therefore it is thought that purinergic signalling has a function in the mechano-sensory transduction in the bladder (32, 33).

The role of ATP in bladder pain has been studied using the mouse model. P2X3 receptor knock out mice have displayed reduced pain related behaviour in response to chemical irritation of the bladder with formalin and ATP injection. Furthermore, these mice also exhibited marked bladder hyporeflexia characterised by a decreased micturition frequency and an increased bladder capacity under normal bladder pressures (34). Vlaskovska et al also found that the P2X3 knockout mice, ATP release was proportional to the extent of bladder distension with distension resulting in a progressive increase in the activity of afferent nerves (35). Intravesical ATP, in the presence of a compromised urothelium, has also been shown to induce detrusor overactivity via the P2X receptor (36).

During inflammation, ATP is also thought to play a role with an increase in ATP release. In patients with interstitial cystitis, increased ATP secretion has been found as well as an augmented release of ATP in response to stretch (37).

It has therefore been suggested that Inflammation of the urothelium may play a part in the pathophysiology of OAB by not only disrupting the integrity of the urothelial barrier but also by leading to an increased release of ATP by the urothelium. An aberrant ATP response induced by an inflammatory reaction may also play a role in the pathophysiology of OAB by increasing sensory nerve excitation.

1.4 Diagnosis of OAB and Exclusion of UTI

When assessing all patients with LUTS it is universally agreed that the mandatory first step is the exclusion of UTI. In patients presenting with acute frequency and dysuria, diagnosis is based on history alone. However, in patients presenting without dysuria we rely on investigations to help exclude UTI. Internationally and nationally these include the quantitative clean-catch midstream urine culture, which is ubiquitous as the gold standard for diagnosis, as recommended by NICE (2015) in the UK. NICE and the International Continence Society (ICS) (2002, 2010) also recommended the use of surrogate markers of infection which include microscopy of urine and the use of urinary dipstick tests.

Published guidelines across Europe, USA and the UK reveal significant discrepancies in the choice of a quantitative threshold used to define significant bacteriuria. The clean-catch, midstream urine (MSU) sample culture in the UK and Europe commonly uses the Kass (1957) criterion of 10^5 colony forming units (cfu) ml^{-1} of a single species of a known urinary pathogen (38, 39). Kass drew these data from 74 women with acute pyelonephritis and 337 normal controls, a select sample unrepresentative of wider lower urinary tract symptoms (LUTS). Despite its limitations, this criterion has become a ubiquitous reference standard and has been challenged by several groups (40, 41). The Associate of Urology (EUA) guidelines for urological infections emphasise that no single threshold can be applied in all clinical situations. The urinary dipstick tests for nitrite and leucocyte esterase, which have been validated against the Kass criterion, have also recently been found to be unreliable (42-45).

1.4.1 Quantitative MSU culture

The quantitative microbiological bacterial culture remains the reference gold standard in diagnosis of significant bacteria. Currently in the UK the proposed threshold that discriminates normal from pathology is 10^5 cfu ml^{-1}. This threshold is based on work by Kass with data from 74 patients with acute pyelonephritis and 334 asymptomatic controls. He proposed that bacterial growth of greater than 10^5 cfu ml^{-1} of a single known urinary pathogen was significant of urinary tract infection in this group of patients. This work has formed the basis of the current quantitative threshold used to exclude UTI in patients presenting with LUTS. There has since been other work in patients presenting with classic symptoms of acute cystitis with sudden onset of pain, frequency and urgency. It was found that a threshold of 10^5 cfu ml^{-1} missed nearly 50% of genuine coliform infections. It was proposed that in patients with symptoms of acute cystitis, bacterial growth of greater than 10^2 cfu ml^{-1} of a known urinary pathogen was a more appropriate threshold in this group of patients (40, 46). Unfortunately however much of this work has been overlooked and many laboratories still use the threshold of 10^5 cfu ml^{-1} when assessing urine samples from patients with lower urinary tract symptoms.

In clinical medicine, to aid diagnosis we frequently use abolute terms to desribe the existence or absence of disease. However nature and biology do not allow such distinct entites and commonly disease manifests across a spectrum (2). Diagnostic test performance is influenced by spectrum bias where the test properties are qualified by the spectrum of disease under scrutiny (47). The quantitative routine MSU is an example of this. In patients describing symptoms of acute frequency and dysuria the diagnosis of UTI is not challenging and should be based on history alone. However in patients presenting with non-dysuric symptoms we still rely on the use of current

investigations to help exclude urinary infection. It is now widely appreciated that in patients with lower urinary tract symptoms, a midstream urine sample that is reported as negative based on the 10^5 cfu μl^{-1} threshold does not exclude significant UTI (40, 45, 48-51). The use of test boundaries and a dichotomous threshold to define the presence/absence of disease is artificial, and distorts our understanding of the underlying pathology.

The work by Kass also only addressed pathogenic coliform organisms, and mixed growth was thought to be likely due to contamination and was dismissed. There is now evidence to support polymicrobial infection in human disease, with work exploring this in the bladder (41). The culture methods used today also assume dominant pathogenicity from the Enterobacteriaceae species, notably *E coli*. Because of this assumption, the MSU culture is performed on a selective medium (selective chromogenic medium) for Enterobacteriaceae under aerobic conditions. No anaerobic bacteria are sought and some aerobic bacteria can be missed because of the choice of medium. Whilst this might be appropriate for diagnosing acute infection, there is no evidence that the same bacteria are implicated in chronic infections or patients presenting with non-dysuric lower urinary tract symptoms such as in the overactive bladder. Thus, there are no data to inform the selection of a suitable gold standard for diagnosing UTI in this context.

1.4.2 Microscopy for pyuria

Microscopy of urine for the detection of urinary leucocytes is widely used as a surrogate marker of urinary infection. Many laboratories also use it as a screening tool for all urine samples received and only those deemed to have a significant pyuria are cultured. This technique stems from Dukes work in 1927 (52) which predates Kass' work. His

work reviewed midstream urine samples from 300 asymptomatic volunteers and found a mean leucocyte counts of 1.6 wbc μl^{-1} in males and 5.4 wbc μl^{-1} in females with a range of 0-50 wbc μl^{-1} (52). From this, he proposed an arbitrary cut off of <10 wbc μl^{-1} as the limit for normal pyuria. As a consequence we now use a microscopic pyuria ≥10 wbc μl^{-1} to suggest urinary infection. The work by Dukes is open to similar criticism to Kass' work of spectrum bias. Here a healthy population has been used to define disease in a symptomatic population. In addition the data from Dukes work shows a wide range of pyuria and hence the data would be skewed and not normally distributed. Therefore use of the median would have been a more appropriate measure of central tendency and had that occurred, zero pyuria would have been promoted as the normal state. The samples collected in Dukes' work were also voided samples and without careful sampling may be subject to contamination. Studies comparing catheter sampling do not report any differences in leucocyte excretion between the sexes.

Microscopic pyuria is commonly used to exclude infection and many studies have reviewed its performace against quantitative bacterial culture. Some studies have however found that those with symptoms of acute UTI were culture negative, against a threshold of $\geq 10^5$ cfu ml^{-1} as diagnostic of infection, nearly half demonstrated leucocyte excretion of ≥10 wbc μl^{-1} (53, 54). These data support pyuria excretion ≤10 wbc μl^{-1} as a common finding amongst women with acute cystitis symptoms, and questions the microbiological definition of UTI, long before Kass' work was re-evaluated. This history illustrates the error of propositions being accepted as explanatory in the absence of empirical support.

There are limited data evaluating the role of microscopic pyuria as a surrogate marker of infection in patients with non-acute symptoms. In patients with painless LUTS

microscopic pyuria has been reported to have a sensitivity of 56% (95% CI 46-60%) and specificity of 72% (95% CI 67-76%) for midstream urine (MSU) cultures at $\geq 10^5$ cfu ml^{-1}, and 66% (95% CI 54-77%) and 73% (95% CI 69-78%) respectively for catheter specimen of urine (CSU) culture at $\geq 10^5$ cfu ml^{-1} (55). In patients presenting with non-acute symptoms Kupelian et al found the positive predictive value (PPV) and negative predictive valve (NPV) of pyuria as a surrogate marker of UTI to be 0.40 (CI 0.37-0.43) and 0.75 (CI 0.73-0.76) respectively. They found that 40% of the inflammatory signal was lost by 4 hours after sample collection which is a common delay between patients providing samples and time taken to arrive at laboratories for analysis (56). Given that microscopy of urine is performed by laboratories so that only those with ≥10 wbc μl^{-1} require culture samples may be wrongly dismissed as negative for infection.

1.4.3 Urinary dipstick

Urinary dipsticks are commonly used in clinical practice to help in the diagnosis of urinary tract infection. They were introduced as part of 'point of care testing' to aid in diagnosis of UTI and reduce the need for urine microscopy and urine culture and allow for earlier treatment. Modern reagent strips are able to provide information on a variety of physical and biochemical variables. The principle measure of UTI includes the presence of leucocyte esterase and nitrites. Leucocyte esterase is an enzymic leucocyte product and so a potential measure of pyuria. The Nitrite test uses the Greiss reaction to detect a nitrate reduction product of some uropathogenic bacteria. The chemical basis of this reaction is that in an acid environment nitrite reacts with an aromatic amine (sulfanilamide) to form a coloured diazonium salt that in turn reacts with hydroxybenzoquinolone to provide a pink colour. It requires the presence of bacteria that can convert nitrate in the urine to nitrite (57).

The International Consultation on Incontinence guidelines on LUTS emphasises the need to exclude UTI and recommends dipstick testing as a screening method (58). NICE also recommends the use of urinary dipstick for screening patients with lower urinary tract symptoms (59). However, there are no published data to support dipstick as a valid screening tool for patients with non-acute, non-dysuric symptoms. On reviewing the literature there is considerable evidence to question the accuracy of urinary dipsticks (42, 43, 60, 61). The sensitivity of leucocyte esterase has been suggested to be around 60% and specificity of around 70%. The sensitivity of nitrite is approximately 18% - 50%, with reported specificities of 90% (42, 61). Again, these estimates relate to patients with acute symptoms, with positive reference cultures at $\geq 10^5$ cfu ml^{-1}.

1.5 Acute Urinary Tract Infection

1.5.1 Aetiology and Pathophysiology UTI

Urinary tract infection is one of the commonest bacterial infections worldwide. The American Foundation for Urological Diseases reports that acute bacterial cystitis affects 8–10 million Americans a year and most of these patients are female with an annual cost of $2.14 billion(10, 62). One in three women will develop a UTI requiring antibiotic treatment by age 24, and 50% experience at least one during their lifetime (62, 63). It has been suggested that predisposing factors include urinary tract obstruction, incomplete voiding, and aberrant structural anatomy. Additional risk factors include prior history of UTIs, vaginal intercourse within the past two weeks, use of contraception with spermicide, low vaginal oestrogen level, and individual genetic background (10).

The incidence of cystitis is significantly higher in women than men, possibly the result of anatomical differences. The shorter female urethra might facilitate bacterial transit from the urethral opening to the bladder and colonisation of the vaginal introitus by gastrointestinal pathogens, which are usually part of the normal faecal microbiota (50). This could also be influenced by sexual activity. The role of the normal vaginal microbiota in the defence against genital colonisation with potentially pathogenic adhering *E. coli* has been suggested in some studies. The reported vaginal colonisation rate of *E. coli* varies from 6 to 26% (64).

Defensive properties of the commensal microbiota against colonisation by bowel organisms include the production of inhibitory substances against pathogens, colonisation of epithelial surfaces, and competition with potential pathogens for sites of adhesion. The relationship of vaginal *E. coli* load with phases of the menstrual cycle also indicates hormonal influence on vaginal colonisation with *E. coli* (65). Local trauma, such as sexual intercourse or urethral massage, promotes invasion of the urinary tract. A vaginal pH of 5 or less protects against vaginal colonisation and urogenital infections. Lactobacillus species usually colonise the vagina and generate an acidic vaginal pH, which interferes with the adhesion of *E. coli*, one of the most common uropathogens in otherwise normal women. It is also likely that use of soaps to clean the genital tract alters the pH and normal microbiota. In addition, the use of diaphragms, cervical caps, or spermicides for contraception is associated with a higher incidence of UTI. Most spermicides contain Nonoxynol-9, which appears to be active against hydrogen producing *Lactobacillus* in the vagina (64).

In females with recurrent UTI, hormonal factors are thought to influence bacterial attachment to epithelial cells. Genitourinary mucosal cells have oestrogen receptors

(66). Adherence changes during the menstrual cycle and is maximal during peak oestrogen stimulation. Oestrogen deficiency in postmenopausal women is associated with a higher risk of UTI. The suggested mechanism is that mucosal atrophy leads to lower levels of lactobacilli in the vaginal microbiota with increases in vaginal pH, promoting colonisation by Enterobacteriaceae. The Cochrane review 2008 found two studies showing vaginal oestrogens to be superior to antibiotics for treatment (67, 68). Raz et al reported both a lower vaginal pH in the oestrogen treatment group and a higher proportion of vaginal Lactobacillus spp. as compared to controls (68).

1.5.2 Uropathogens

There are many studies reviewing the organisms responsible for urinary tract infection. Much of this work has been from patients with acute UTI or pyelonephritis. Known uropathogens have been described but much of this work has used quantitative thresholds and hence this will contribute to our current understanding of urinary ecology. The dismissal of polymicrobial cultures as likely contamination, and uncertainty relating to the uropathogenic potential of specific genera/species, also question established thoughts on uropathogens. Whether the bacterial ecology of urinary tract infection differs across the spectrum of clinical syndromes is unknown.

The ECO·SENS project (60) was large study that included all community patients with symptoms of acute cystitis and used a threshold for women with acute cystitis with a positive growth reported as growth $\geq 10^3$ cfu ml^{-1}. The study reported all uropathogens and included polymicrobial growth of two isolates. Uropathogenic *E.coli* (UPEC) was the commonest bacterium accounting for approximately 75% of samples. Other Enterobacteriaceae, including *P. mirabilis, Klebsiella, Enterobacter,* and *Citrobacter*, were reported in 15%; *Staphylococcus saprophyticus* in 5%, with other *Staphylococci*

and *Enterococci* in the remaining 5%. Within the literature *Group B streptococcus* has also been associated with UTI in pregnancy and *Proteus* and *Pseudomonas* with hospital acquired UTI (69).

There is limited data on the presence on anaerobic bacteria in the urinary tract. In one large study only 1.3% of samples grew anaerobic organisms, predominately *Lactobacillus spp* and *Clostridium* (70). Similarly another study found positive growth of anaerobic bacteria in only 0.03% of samples, with positive growth as more than 10^8 cfu ml^{-1} of a single organism, which may explain the lower prevalence (71). On reviewing the literature it can be suggested that anaerobic bacteria may have a role in a small number of urinary tract infection. However the limited date may be a reflection of the difficulty in culturing these bacteria and that Anaerobic culture methods are not routinely used clinical practice and that there is need for further work to reach a probably conclusion.

Atypical bacteria such as *Mycoplasma spp, Ureaplasma spp* and *Chlamydia spp* have also been implicated in causing UTI. However again these were from smaller studies where there was also presence of uropathogenic bacteria. Hence it is difficult to ascertain what role these organism my play in the generation of symptoms (72, 73).

1.5.3 Bacterial urothelial invasion and intracellular growth

For pathogen host interaction to occur, bacteria must be able to attach and adhere to host tissues. Much of the work has centred on the murine model of acute UTI with UPEC (74) (50). The urothelium has been described to have a surface layer of umbrella cells, which act as a protective barrier. In the murine model of acute model, colonisation of the bladder by UPEC is dependent upon the mannose-binding adhesion,

FimH, at the tip of type 1 pili (75). FimH binds to uroplakins on the superficial umbrella cells of the urothelium, mediating colonisation and triggering subsequent bacterial internalisation into the bladder epithelial cells (76). Once inside the epithelial cells, UPEC bacteria are protected from the host's innate immune defences. This allows the bacterium the ability to replicate rapidly forming biofilm-like intracellular bacterial communities (IBCs) (74). Similarly to extracellular biofilms, IBC formation is transient and terminates in a dispersal stage, during which bacteria escape the infected host cell, spreading to neighbouring (naive) host cells, where the IBC cycle can be repeated (77). Numerous host defences against this process, including inflammasome activation and programmed urothelial exfoliation have been suggested (76). UPEC strains have been shown to form IBCs in murine mouse models. This allows UPEC to persist and form quiescent intracellular reservoirs (QIRs) or the development of chronic cystitis (77).

1.5.4 Host defence against infection

The first defences against bacterial infection and adhesion to the urothelium are the physical and chemical properties of the urine. Invading bacteria stimulate micturition. Voiding washes out bacteria from the bladder and urethra and urine dilutes the bacterial load. Bacterial growth is impeded by low pH and the high urea and organic acid concentrations and the extremes of high and low osmolality discourage less adapted bacteria (77). Nevertheless, aggressive bacterial species have evolved mechanisms to overcome these obstacles. There are a number of interrelated anatomical/ physiological factors that influence the maintenance of bacterial numbers in urine once infection is established. These include the flow rate of urine into the bladder; the volume of residual urine remaining in the bladder after micturition and the frequency with which micturition occurs.

The flow rate of urine into the bladder is inevitably variable, but averages at about 1 ml/min during the day and at about 0.25 ml/min during the night (78). The flow rate is increased by high fluid intake and diuretics, so the urine produced may be dilute and less suitable for supporting bacterial growth. Providing the urine is not carrying bacteria from a focus of infection in the kidney, the influence of urine flow on bacterial numbers in bladder is a dilution effect. However, this dilutional effect also influences the immune response, by diluting immune cells at the site of infection. Furthermore, post micturition residual urine can vary enormously in the infected bladder (79) . The interval between episodes of micturition will fluctuate during the day and vary with fluid intake. Most people micturate only once, or not at all, during the night, but urinary infection may alter this habit. In acute cystitis frequency of micturition is a common presenting symptom. This may be invoked as a natural defence mechanism until the resolution of infection. In addition, higher residual volumes are also associated with urinary tract infection, particularly in the postmenopausal group.

As a result of normal kidney function, the variability in the chemical characteristics of urine such as pH and osmolality can promote or suppress bacterial growth. Any reduction in the bacterial growth rate will be to the patient's advantage, but this does not necessarily equate to elimination of infection. The situation may be complicated by the fact that the bacteria may fix themselves to the surface of the bladder mucosa or reside in foci within the urinary system where they are unaffected by hydrokinetic forces.

The importance of 'bound' organisms in the persistence of infection has been investigated, through mathematical modelling, by Mackintosh et al. (80). They calculated that if 10 'bound' bacteria continuously seed the urine with fresh organisms,

infection would be maintained at around the 10 bacteria per ml level, even in the most favourable washout conditions in which a patient with a normal residual bladder volume micturates every hour. This synthetic data are supported by clinical studies which have shown that infection will persist in a proportion of women despite adequate diuresis and any impairment of the efficiency of the washout mechanism will exacerbate the situation (81).

1.5.5 The inflammatory response to UTI

It was initially thought that urinary defence may be dependent on specific soluble epithelial cell-derived mediators such as Tamm-Horsfall protein (THP), soluble IgA, Lactoferrin, lipocalin, antimicrobial peptides such as α- and β-defensins and cathelicidin that have evolved under microbial pressure to combat uropathogenic bacteria (82). These identified molecules may exert multiple functions including mechanical elimination of bacteria and inhibition of invasion. Microbes evade these early defences and adhere and initiate an immune response.

Urothelial damage by infection leads to the release of inflammatory mediators including platelet derived growth factor (PDGF), tumour necrosis factor-α (TNF-α), interleukin 6 (IL6) and interleukin 8 (CXCL 8). Infection by *UPEC* has been well studied in which a rapid cytokine response has been seen by adhering P or type-1 fimbriated *E.coli* (83-85). In urinary tract cell lines, epithelial cell activation by fimbriated *E. coli* requires primary recognition receptors for fimbrial adhesins and Toll-like receptor 4 (TLR4) for transmembrane signalling (86). Human urinary tract epithelial cells express both glycosphingolipid and mannosylated surface glycoprotein receptors, which recognize the P fimbrial adhesions and the type1 fimbriae, respectively (83, 87). Bacterial components bind to Toll-like receptors (TLR) and thereby activate an intracellular innate

immune cascade. A key player in the bladder is TLR-4, which is assisted by CD14 to bind lipopolysaccharide (LPS) (88). TLR-4, -2, and -5 binding activates the MyD88 pathway, which ultimately leads to NF-κβ transcription and proinflammatory cytokine production. The six key cytokines that are induced are notably IL-6, CXCL8 (IL-8) as well as ICAM-1, CXCL-1, CXCL-5 and CXCL-6. IL-6 may cause fever and trigger the acute-phase response, while chemokines such as IL-8 recruit inflammatory cells to the site of infection (88). IL6 has also been implicated in a chronic inflammatory response (89, 90).

Urinary cytokines are elevated in patients with UTI and epithelial cells have been identified as early producers of cytokines in the murine UTI model (91) (92, 93) (94). Uroepithelial cell lines of bladder and kidney origin constitutively make IL-6 and respond with elevated IL-6 production to exogenous stimuli like bacteria or cytokines (91, 95, 96). Studies of patients with UTI have shown rapid increases in urine IL6 levels after the onset of infection or instillation of bacteria into the urinary tract but serum IL6 levels are only elevated in those with acute pyelonephritis (97-99). Through the release of cytokines, epithelial cells establish a network with local and distant host cells (95). Cell recruitment follows after the secretion of chemokines like IL-8. Studies have shown that IL-8 is present in epithelial cells of the healthy urinary tract and that IL-8 is secreted in response to infection. IL-8 then returns to non-detectable levels after treatment, and is absent from normal urine (100).

1.6 OAB and the role of infection

In assessing anyone who presents with LUTS a mandatory step is the expulsion of UTI. Routine urinalysis and culture are unreliable in excluding infection in this group of patients and hence significant infective pathology may have been missed.

In patients with OAB symptoms there are studies that suggest increased inflammatory activity and bacterial colonisation, not seen in asymptomatic control subjects. This evidence postulates that bacterial infection and associated urothelial inflammation may be responsible for the generation of chronic lower urinary tract symptoms. Khasryia et al found that patients with chronic lower urinary tract symptoms have higher urinary pyuria and microbial growth when compared to controls (55). Bacterial strains from symptomatic patients were also shown to invade urothelial cell lines whilst bacteria isolated from controls did not. It was hypothesised that patients with chronic lower urinary tract symptoms have intracellular bacterial colonies that are responsible for an inflammatory response and generation of symptoms. This group of patients also showed increase urothelial cell shedding. These cells were harvested from the urine and underwent an antibiotic protection assay where the cells where incubated with a lethal cocktail of antibiotics to kill any extra cellular bacteria. On lysis of these cells after 24 hours there was an increase in bacterial growth suggestive that these urothelial cells were harbouring intracellular or cell associated infection, which was not killed by extracellular antibiotics.

Horsley et al 2013, have also suggested the presence of intracellular bacterial colonies and bacterial pods protected by biofilms, in urothelial cells from patients with chronic lower urinary tract symptoms (101). The authors used methods including fluorescent confocal microscopy and electron microscopy to confirm that these were intracellular bacterial communities.

There is also histological evidence to support infection in patients with OAB and elevated pro-inflammatory urinary cytokine release. Lunawat et al found that in all 61

patients with OAB and pyuria, but negative routine microbial culture, bladder biopsies manifested all the uroepithelial features of chronic cystitis; no features of inflammation were identified in control samples (102). Vijaya et al found increased bacterial growth on culturing bladder biopsies obtained at cystoscopy from patient with overactive bladder (103). The routine MSU in all these patients was negative but culture of bladder biopsies showed significant microbial growth and elevated urinary NGF levels as compared to controls. These patients were then treated with antibiotics and the urinary NGF levels were shown to decrease after treatment.

1.6.1 OAB and urinary cytokines

There are limited and conflicting data on the expression of inflammatory mediators in OAB. Other lower urinary tract syndromes such as, interstitial cystitis and painful bladder syndrome have been linked to an inflammatory aetiology. Interstitial cystitis and the painful bladder syndrome are associated with increased mast cells on bladder biopsy and raised inflammatory markers in the urine including histamine, methylhistamine and IL6 (104). Erickson et al. found elevated levels of IL6 and epidermal growth factor (EGF) in patients with interstitial cystitis (105). Bouchelouche et al. showed that IL-1β and TNFα stimulate the secretion of IL6 in cultured human detrusor smooth muscle cells (106, 107). The regulatory effect of cytokines in inflammation has been shown in animal models, particularly the stimulation with bacterial endotoxin lipopolysaccharide, leading to the secretion of IL6. Heinrich et al showed an upregulation in the gap junction proteins connexin 43 and 45, secondary to stimulation by IL6 (108). They suggested that this modification of cell-cell communication by IL6 could be pivotal in the pathophysiology of the overactive bladder and interstitial cystitis. Conversely, Ghoniem et al have shown a down regulation of IL6

in the urine of patients with OAB, however, they do not describe the minimum level of detection of the cytokine in their micro-array (109).

Recently, there has been much interest in other inflammatory markers in OAB, particularly for use as biomarkers of the disease. Neurotrophins such as Nerve growth factor (NGF) and Brain derived growth factor (BDGF) and prostaglandins have been studied in OAB (110). Tyagi et al. studied IL-5, IL6, IL-10, IL-12p70/p40, IL-1 receptor antagonist (IL-1Ra), Epidermal growth factor (EGF) and soluble IL-2 receptor a (sIL-2Ra) in the urine of patients diagnosed with OAB compared to control volunteers. They found a significant increase in IL-10, IL-12, EGF and IL-12p70/p40. They suggested a relationship with inflammation in OAB, possibly secondary to irritation or stress (111). Increased urinary CXCL 8 has been shown in interstitial cystitis but not in the overactive bladder (86, 99, 112).

1.7 Hypothesis and Aims

When assessing anyone who presents with lower urinary tract symptoms, excluding a urinary tract infection is key. However there is substantial evidence that refutes the ability of current diagnostic tests to detect and exclude UTI. Therefore the role of infection in the generation of LUTS merits scrutiny. It has been suggested in epidemiological studies that advancing age and female sex may be associated with bacteriuria and pyuria, which is thought to be in the absence of infection as measured by current standards (113-115). There is however some data to suggest bacterial infection, urothelial inflammation and immune activation in patients with OAB symptoms that is undetected by routine tests (51, 102). Observational data also suggest a response to antibiotic treatment in patients with OAB, although further data are

awaited. However, these studies can be criticised due to a younger control population, use of limited of markers and small sample size. There is therefore a need for future studies to match patients and control subjects for key demographic characteristics. In addition it would be important to evaluate the presence and role of infection in patients presenting with overactive bladder. Prospective data evaluating the interaction between symptoms, bacterial ecology and urothelial inflammatory response would add to support a causal relationship.

1.7.1 Hypothesis to be tested

Bacterial infection of the lower urinary tract goes undetected by routine diagnostic testing and contributes to the generation of LUTS in patients with symptoms of OAB.

1.7.2 Thesis Aims

1. To explore the urinary inflammatory response in patients with symptoms of overactive bladder.

2. To determine the relationship between urothelial inflammation, manifested by pyuria, epithelial shedding and microbial growth, and symptom variation in patients with symptoms of OAB.

3. To determine the urinary cytokine and inflammatory response in relation to symptom exacerbation in OAB.

1.7.3 Thesis Outline

Chapter 1 provides a comprehensive introduction to urinary tract infection and an overview of its role in LUTS and OAB. The common processes and methodologies used in this research have been described in chapter 2. Chapter 3 is an evaluation of urinary ATP in relation to infection and inflammation in patients with LUTS. Chapter 4 is a review of key inflammatory cytokines in patients with symptoms of OAB as compared to asymptomatic controls. Longitudinal studies exploring the role of infection and inflammation in patients with OAB symptoms has been addressed in Chapter 5 and 6. Chapter 7 is a critical discussion of the overall work, conclusions and contribution to the scientific literature.

2 Chapter 2 – Patient recruitment and common methods of measuring urinary infection

2.1 Ethical approval

Studies included in this work had ethical approval by the National Research Ethics Service (NRES) - East Central Research Ethical Committee 1 (REC Reference number 11/H0721/7).

2.2 Patient recruitment and consent

Patients were recruited from urological clinics at the Department of Medicine, University College London Archway Campus. The patients who expressed interest were given written information regarding the study and then contacted two weeks later and if agreeable, invited for an initial visit where written informed consent was obtained. Control subjects were recruited from the staff at the Whittington hospital and University College London.

2.3 Data management and protection

Study data were stored in the Department of Medicine, UCL Archway Campus, as per Good Clinical Practice (GCP) guidance. All samples and recoded data were identifiable by study numbers and participant initials. Any patient identifiable data was stored on only a secure NHS database, which was protected by encryption and daily backup.

2.4 Statistical methods

IBM® SPSS® Statistics 20 (IBM, New York, USA) was used for statistical analysis. Data were described using standard descriptive statistics. Specific statistical analysis for each study has been described in each relevant section.

2.5 Study design

Prospective observational cross-sectional and longitudinal cohort studies were conducted using standard methods. Patient and control groups were matched by key demographic characteristics.

2.6 Study groups

Subject Inclusion Criteria

1. Adult Women aged >=18 years
2. Able to complete a symptom questionnaire
3. Diagnosed with OAB with or without pyuria (≥ 1 wbc μl^{-1})
4. Post-menopausal women or women using adequate contraception

Subject Exclusion Criteria

1. Age less than 18 years
2. Inability to consent
3. Pregnant women or women planning to conceive
4. Patients with concurrent illnesses that in the opinion of the investigator were likely to compromise the validity of the data

The study groups were

1) Female adult patients with OAB (1), describing:

a. Urgency, the sudden compelling desire to urinate, a sensation that is difficult to defer.
b. Urinary frequency, voiding eight or more times in a 24-hour period.
c. Nocturia, the need to wake one or more times per night to void.
d. They may or may not experience urinary incontinence.
e. With or without pyuria (≥1 wbc μL^{-1}) and/or a history of acute symptom exacerbations.

2) Female adults without OAB and without other LUTS.

2.7 Symptom collection

2.7.1 ICIQ Questionnaires

The International Consultation on Incontinence Questionnaires (ICIQ) were selected to evaluate symptomatology (19). The ICIQ-LUTS questionnaire is a well-validated tool and was selected to quantify and characterise symptoms and to assess patients' perception of bother by such symptoms. The questionnaire consists of a total of 12 questions; four questions on filling, three on voiding and five questions on incontinence. Each question is scored 1–4; thus, range of overall scores from 0 to 16, 12 and 20 for filling, voiding and incontinence scales, respectively. The overall score is 0 to 48 with higher scores indicating greater impact of individual symptoms for the patient.

2.7.2 Whittington Urgency Score

The symptoms of urinary urgency were measured in detail using a validated tool. The Whittington Urgency Score is a ten-item scale completed by participants that records characteristics and the degree of urinary urgency (116). The questionnaire has been fully validated (116, 117).

2.7.3 Whittington Pain Score

The Whittington Pain Questionnaire, developed from a large symptom dataset provided by patients with interstitial cystitis (IC), was selected for use in these studies. It is a validated, eight-item scale which records the most prevalent dysaesthetic/pain symptoms associated with the lower urinary tract (20).

2.8 MSU sample collection

Participants were asked to provide midstream clean-catch method. Patients were given verbal and written instructions (118) to try and avoid contamination. Participants were instructed to first clean their hands, and clean the genital area with an antibacterial wipe prior to sample collection. Female subjects were instructed to hold the labia apart and cleanse the genial region from front to back with an antibacterial wipe. Uncircumcised males were asked to retract the foreskin and cleanse the glans. Participants were then asked to begin urinating. A sterile bowl was put into the continuous stream to collect the middle part of the void and then removed. This continuous stream of urine, as opposed to stopping and starting, reduced contamination from the perineum. The urine was decanted into three 30 ml sterile universal specimen tubes.

2.9 Urine dipstick testing

Multistix® 8 SG reagent strips were used to test urine sample and analysed using the paired with a Clinitek® Status analyser (Siemens, Munich, Germany). Leucocyte esterase was reported as: 'negative', 'trace', 'one 1 +', 'two 2+', or 'three 3+'; nitrite was reported as 'negative' or 'positive'.

2.10 Cytological assessment

2.10.1 Inflammation: Microscopy for pyuria

Fresh urine samples were collected as described above and immediate microscopy was performed on fresh unspun, unstained urine. A disposable pipette was used to place a drop of urine in the filling chamber of a Neubauer haemocytometer and covered with a glass cover slip. Olympus CX41 light microscope (x200) (Olympus, Southend-on-Sea, UK) was used to analyse the sample. The leucocyte count was enumerated using a standard operating procedure in triplicate. All three measures were recorded and the mean value calculated.

2.10.2 Immune response: Urothelial cell shedding

Fresh, unspun, unstained urine samples were analysed immediately after sample collection. A disposable pipette was used to place a drop of urine in the filling chamber of a Neubauer haemocytometer and covered with a glass cover slip. Olympus CX41 light microscope (x200) (Olympus, Southend-on-Sea, UK) was used to analyse the sample. The epithelial cell count was enumerated using a standard operating procedure in triplicate. All three measures were recorded and the mean value calculated.

2.10.3 Bacterial colonisation: Urothelial clue cell analysis

Urine samples were processed within I hour of sample collection, and were refrigerated at 4°C until assessment. A collection chamber consisted of a single channel cuvette and retainer, a Shandon filter card (Fisher Scientific, Loughborough, UK), and a Superfrost Ultra Plus glass microscope slide. 80 µl of urine was transferred into the collection chamber for centrifugation. The sample was spun at 75 g for five minutes in a Shandon Cytospin 2 cytocentrifuge (Thermo Scientific, Basingstoke, UK). The cellular components of the sample formed a visible deposit on the slide. A hydrophobic barrier pen (Vector Laboratories, Peterborough, UK) was used to outline the area and create a barrier.

The cells were then fixed by adding 100 µl of 4% formaldehyde (Thermo Scientific, Fisher Scientific) in PBS at room temperature for 15 minutes. The formaldehyde was aspirated and the preparation washed three times with PBS at 5 minute intervals. The cell membranes were stained with wheat germ agglutinin (WGA) conjugated to Alexa Fluor 488 (Invitrogen). Wheat germ agglutinin stock solution (1.0mg/mL) was prepared by dissolving 5mg of Alexa Fluor 488 WGA conjugate into 5ml of sterile PBS. The stock solution was stored in aluminium foil to protect from light at -20°C for up to 1 month. To prepare the Alexa Fluor 488 WGA conjugate working solution, the stock solution was further diluted with Hank's balanced salt solution (HBSS) minus phenol red (Invitrogen). This gave a working solution of WGA conjugated to Alexa Fluor 488 of 5.0µg/mL and 100µl of this was added to the cell deposit ensuring complete coverage of the cells. The cellular deposit was incubated for 15 minutes at room temperature. The labelling solution was then carefully aspirated and the cells washed 4 times at 5 minute intervals with HBSS. Alexa Fluor 488 excites at a wavelength of 495nm and emits at 519nm, hence the cell membranes appeared green under fluorescent microscopy. The host and bacterial DNA were stained using the DNA stain, DAPI. 100µl of DAPI (4'',6-diamidino-2-

phenylindole, Sigma-Aldrich) at concentration of 1µg/ml in PBS was added to the cellular deposit. The cells were incubated for 15 minutes at room temperature. The DAPI solution was then aspirated and cells washed 4 times at 5 minute intervals with PBS. DAPI solution (1µg/ml) were stored with protection from light at -20°C. The cells were immediately mounted with FluorSave reagent (Calbiochem) and a coverslip carefully applied ensuring no air bubbles. The coverslip was fixed with clear nail varnish and the slide was left to dry for at least one hour with protection from light. DAPI gives mammalian nuclei and bacteria a blue appearance under fluorescent microscopy; it excites at a wavelength of 360nm and emits at 460nm. DAPI is able to label intracellular and extracellularly attached bacteria without the need for permeabilisation. All slides were stored at 4°C in a light-protective box. The slides were examined under a fluorescent microscope Olympus CX41 upright epi-fluorescence microscope (Olympus), at the Department of Medicine, UCL Archway Campus. Images were processed using ImageJ 1.44P and Axiovision Rel. 4.8 software (Carl Zeiss, Cambridge, UK). The proportion of clue cells were calculated by counting the total number of cells present and then the proportion of cells with associated bacteria. Counts were performed in triplicate and an average clue cell proportion recorded.

2.11 Urothelial cytokine response

Urine samples were centrifuged using a Denley BR401 centrifuge (R_{MAX}=140mm) (Denley, Heckmondwike, UK) at 627 *g* for 5 minutes. The supernatant was carefully removed into 20, 1ml freezing microtubes (Sigma-Aldrich) for immediate storage at -80°C. For each experiment a new aliquot of urine was used to ensure that each sample only underwent one freeze thaw cycle. Details of methods used for individual cytokines are described in relevant chapters.

2.11.1 Concentration correction: Urinary creatinine

The measurement of urinary cytokines may be influenced by urinary concentration. Urinary creatinine, a measure of glomerular filtration rate, was used as a correction factor and concentration of cytokines expressed as a ratio of the creatinine concentration. Thirty milliliters of urine in a sterile universal specimen tube was sent to the Whittington Hospital biochemistry laboratory for analysis. Samples were processed immediately upon receipt, or after overnight refrigeration at 4°C. Urinary creatinine was measured using an automated Jaffe technique, in which creatinine and alkaline picric acid produce a red/orange complex measured by spectrophotometry.

2.12 Microbiological assessment

2.12.1 Enhanced Sediment culture

Enhanced sediment cultures were processed within two hours of urine sample collection. Samples were refrigerated at 4°C until processed. All sediment cultures were conducted in the research laboratory at the Department of Medicine, UCL Archway Campus.

2.12.1.1 Preparation and inoculation

Five millilitres of fresh unspun unstained urine was transferred into a sterile centrifuge tube. The sample was centrifuged at 627g for 5 minutes in a Denley BR401 centrifuge (R_{MAX}=140mm) (Denley, Heckmondwike, UK). The supernatant was carefully removed, leaving the urinary sediment. The sediment was resuspended in 400 µl of 1% sterile PBS solution. Four, 1:10 serial dilutions were performed for accurate quantitative bacterial

counting. Serial dilutions were performed by resuspending 100µl of the sediment solution into 900µl of 1% sterile PBS, for a 1:10 dilution. This was then further diluted by taking 100µl of the 1:10 solution and resuspending this in a sterile Eppendorf containing 900µl of 1% sterile PBS for a 1:100 dilution. This was repeated a further two times to produce five sediment suspensions of concentration neat to 10^{-4}.

Chromogenic CPS3 agar plates (bioMérieux, Basingstoke, UK) were used for culture. The plate was divided into 5 subsections and 50µl of the resuspended sediment and serial dilution pipetted and spread onto each area. In addition 50µl of the stock 1% PBS solution was plated onto a Columbia Blood Agar (CBA) plate (Oxoid, Basingstoke, UK). All culture plates were incubated aerobically for 24 hours at 37°C in a CO_2 dependant incubator. If there was growth on the CBA plate, all samples where that stock PBS was used, were discarded as this implied contamination of the stock 1% PBS.

2.12.1.2 Bacterial Quantification

Each bacterial isolate was quantified. No threshold was used to discriminate positive or negative growth. The number of colonies of each isolate was determined in each sector of the CPS3 plate, corresponding to one of five serial bacterial dilutions. The mean colony count from all sectors was calculated.

2.12.1.3 Colour identification of colonies

The CPS3 chromogenic medium allows bacterial identification of uropathogens to genus or species level, dependent on the microbe. The growth of distinct bacteria is colour specific to allow easy and fast enumeration. Colour identification was based on the manufacturer's standardized colour guide. Colour-based identification was

supplemented with Gram staining and rapid biochemical tests for further characterisation. Analytical Profile Index (API) testing could be used when these methods were unable to conclusively identify an isolate.

2.12.1.4 Bacterial identification: Gram Stain

Gram staining was used to determine the morphological characteristics and cell wall composition of bacteria where further classification was required. A single pure colony was picked with a sterile loop and mixed on a glass slide with 20µl of PBS. The solutions were allowed to dry in air. Crystal violet stain (Sigma) was added over the slide. This was allowed to stand for 30 seconds. The slide was then gently rinsed with a stream of water. Iodine solution (Sigma) was added on the smear, enough to cover the slide. This was allowed to stand for 30 seconds. The iodine solution was rinsed off the slide with running water. A few drops of acetone (Sigma) were added so the solution trickled down the slide and rinsed off with water after 5 seconds. A counter stain of 1% neutral red solution carbol fuchsin (Sigma) was added for 30 seconds. The solution was washed off with water. The slide was air-dried after blotting off excess water.

Gram-positive bacteria exhibited a dark purple colour due to their ability to retain crystal violet within their peptidoglycan exoskeleton. In contrast, Gram-negative bacteria were unable to hold crystal violet. Instead, the counter stain dilute carbol fuchsin (pink) was displayed. The morphology was characterised as either rod or bacillus (cylindrical in shape) or coccus (spherical in shape).

2.12.1.5 Bacterial identification: 'Spot' biochemical testing

Rapid reagent testing ('spot testing') using catalase, indole, oxidase, bile esculin and coagulase tests (Remel, Basingstoke, UK) were employed to supplement colour-based bacterial identification.

2.12.1.5.1 Catalyse test

Using a sterile disposable loop, growth from the centre of a colony was transferred to a clean glass slide. 20µL of 3% hydrogen peroxide (Sigma) was added to the colony. The sample was observed for effervescence. A sustained appearance of effervescence was reported as positive.

2.12.1.5.2 Indole test

Indole spot reagent is used to detect the presence of indole, which is one of the degradation products of the bacterial metabolism of tryptophan. It is used to differentiate *E coli* from other bacterial genera such as *Klebsiella, Enterobacter* and *Serratia*. Using a sterile disposal loop, growth from the centre of a colony was transferred into sterile blotting paper containing a drop of indole and observed for 30seconds. A positive result was demonstrated by a blue to blue-green colour, and a negative result was demonstrated by a clear or light pink colour.

2.12.1.5.3 Oxidase test

A solution of tetramethyl-p-phenylenediamine (Sigma) was prepared in 1ml of sterile distilled water. A sterile swab was placed into the solution and soaked. The cotton bud was then touched onto the surface of the test colony. A positive reaction was taken as the development of a purple colour within 20 seconds. *Pseudomonas aeruginosa* (NCTC 10662) was used as a positive control.

2.12.1.5.4 Bile Esculin

Bile Esculin agar, a selective differential agar, was used to confirm the identity of *Enterococcus*. Members of the genus *Enterococcus* are able to grow in the presence of 4% bile (oxgall) and hydrolyzing esculin to glucose and esculetin. Esculetin combines with ferric ions to produce a characteristic black complex, which constitutes a positive result.

2.12.1.5.5 Co-agulase test

The coagulase test was used to differentiate *Staphylococcus aureus* from coagulase-negative *staphylococci*. Two drops of saline were put onto the slide and emulsified with the test organism using a sterile loop. A drop of plasma (rabbit plasma anticoagulated with EDTA) was placed on one of the inoculated saline drop and mixed well and the slide is rocked gently for about 10 seconds. If 'positive', macroscopic clumping was observed in the plasma within 10 seconds, with no clumping in the saline drop. If 'negative', no clumping was observed.

2.12.1.6 Bacterial sub-culture and isolate storage

Each bacterial isolate identified on chromogenic agar was sub-cultured. A single colony of bacteria was plated on a CBA plate using a sterile 1µl inoculation loo to allow pure growth. The plate was incubated for 24 hours at 37°C. Each Bacterial isolate was labelled stored in 2 ml cryopreservation vials (Thermo Scientific) as per the manufactures instructions at -80°C in the Department of Medicine, UCL Archway Campus.

2.12.2 Routine MSU culture

All routine microbiological cultures were undertaken in the Whittington Hospital microbiology laboratory. Thirty millilitres of urine in a sterile universal specimen tube was cultured immediately upon receipt, or after overnight refrigeration at 4°C. Trained biomedical scientists undertook all analyses.

2.12.2.1 Preparation and inoculation

One microlitre of the urine sample was inoculated onto a CPS3 agar plate using a sterile 1μl loop. Inoculation was achieved by streaking the loop across the plate. The culture plate was then incubated aerobically for 24 hrs at 37°C. Bacterial colonies were identified by colour and morphologic characteristics, as described previously. Rapid reagent testing ('spot testing') was employed to supplement colour-based bacterial identification.

2.12.2.2 Bacterial Quantification

Routine culture techniques are semi-quantitative, and bacterial growth is estimated by visual assessment of colony density. A 'positive' culture was defined as the growth of a single recognised uropathogen at $\geq 10^5$ cfu ml^{-1}. Polymicrobial growth above this threshold was reported as 'mixed growth'. Any bacterial growth below 10^5 cfu ml^{-1} was reported as 'no significant growth'.

3 Chapter 3 – Urinary ATP as marker of urinary infection in patients with LUTS – cross sectional data

LUTS is a collective term describing (1) urinary storage problems such as frequency, urgency and urge incontinence described as OAB and the main focus of this thesis; (2) voiding difficulties such as hesitancy, reduced stream, intermittency and incomplete voiding; (3) sensory symptoms that include various experiences of pain; and (4) stress urinary incontinence. There is considerable overlap between these symptoms (Coyne 2009) so that diagnostic categorisation is difficult and it is impossible to consider OAB without reference to the other symptom sets. Part of the process of discerning the dominant problem is the exclusion of urinary tract infection; whatever the symptom mix, it is a mandatory first step in the assessment of all LUTS (119). Whilst acute UTI is not diagnostically challenging, in the case of LUTS without acute frequency and dysuria, exclusion of infection poses a diagnostic challenge.

There are good reasons to scrutinise urinary adenosine-5'-triphosphate (ATP) as a possible surrogate marker of UTI since the reliability of popular diagnostic methods used to exclude UTI have been questioned (40, 42, 45, 120). Published guidelines across Europe, USA and the UK reveal significant discrepancies in the choice of a quantitative threshold used to define significant bacteriuria. The clean-catch, midstream urine (MSU) sample culture in the UK and Europe commonly uses the Kass (1957) criterion of 10^5 colony forming units (cfu) ml^{-1} of a single species of a known urinary pathogen (38). Kass drew these data from 74 women with acute pyelonephritis and 337 normal

controls, a select sample unrepresentative of wider lower urinary tract symptoms (LUTS). Despite its limitations, this criterion has become a ubiquitous reference standard and has been challenged by several groups (40, 41). The European Associate of Urology (EUA) guidelines for urological infections emphasise that no single threshold can be applied in all clinical situations. The urinary dipstick tests for nitrite and leucocyte esterase are also commonly used as a bedside screening test for infection and as a measure of a positive urine culture. However, the use of urinary dipstick has been validated against the urine culture to a threshold of 10^5 colony forming units (cfu) ml^{-1} and following the recent criticism of the Kass criterion, urinary dipstick have also been found to be unreliable (42, 43, 45).

Urinary tract ATP has attracted intense interest in the last 30 years for its pharmacological and pathophysiological associations. There are great hopes that purinergic receptor manipulation might influence detrusor motor function and urothelial afferents (121) achieving therapeutic benefit for patients with OAB. ATP is an important urothelial cell distress signal (122) and is released by inflammatory cells and bacteria (123). Urinary tract infection, featuring bacterial invasion, urothelial distress and an innate immune response involving recruitment of inflammatory cells, should be associated with increased urinary ATP levels. Indeed, high levels of ATP have been detected in the urine of patients with interstitial cystitis and acute UTI with a positive urine culture (124). Increased ATP has also been shown to be released from cultured urothelial cells infected with uropathogenic E.coli (UPEC) (125) and UPEC also produce ATP when cultured in vitro (126). It has been postulated that ATP may reflect microbial biomass and hence ATP increases as the amount of bacteria present increases. Currently ATP levels are used widely in the food, water and sanitation industry as a measure of bacterial contamination (127).

3.1 Hypothesis

Given the problems with current tests developing alternative diagnostic assays is a high priority. I sought to scrutinise the performance of urinary ATP as a potential surrogate marker for urinary infection in patients with lower urinary tract symptoms.

The hypothesis to be tested was:

1) Patients with OAB show a higher urinary inflammatory signal as compared to controls with higher concentrations of urinary ATP.

2) Urinary ATP has the potential as a surrogate marker of urinary infection in patients with chronic lower urinary tract symptoms.

3.2 Study Design

The study was divided into two parts; (1) a clinical experiment that evaluated urinary ATP in patients with LUTS and controls, comparing urinary ATP with symptoms, microscopic pyuria and urine culture results; and (2) a laboratory experimental series that explored the factors that could influence sample collection, storage and preservation. As urine contains native ATPase activity, the time-decay curve of urinary ATP from collection to processing was evaluated. Boric acid crystals, which are commonly used as a urinary preservative, have been shown to prevent microbial swarming (128) and boric acid has a preservative influence on white cells (129). The effect of the use of urinary preservative boric acid, storage temperature and the effect of centrifugation on urinary ATP concentration was studied.

3.3 Ethical Approval

All of the studies included in this work were subject to approval by the National Research Ethics Service (NRES). This study was approved by East Central Research Ethical Committee 1 (REC Reference number 11/H0721/7).

3.4 Inclusion and exclusion criteria

Subject Inclusion Criteria

5. Adults aged >=18 years
6. Able to complete a symptom questionnaire
7. Diagnosed with LUTS with or without pyuria (≥ 1 wbc μl^{-1})
8. Post-menopausal women or women using adequate contraception

Subject Exclusion Criteria

5. Age less than 18 years
6. Inability to consent
7. Pregnant women or women planning to conceive
8. Patients with concurrent illnesses that in the opinion of the investigator are likely to compromise the validity of the data

There were two study groups 1) adult patients with LUTS and 2) control group of adults without any LUTS.

3.5 Study Population

3.5.1 Recruitment

Patients with lower urinary tract symptoms and healthy volunteers were recruited as described in chapter 2.

3.5.2 Consent

Written consent was obtained from all participating individuals as described in chapter 2.

3.6 Safety considerations

There were no safety considerations for patients providing MSU samples only.

3.7 Study Method

All participants provided clean catch MSU samples and immediate microscopy for pyuria and epithelial cells was performed. Aliquots of spun urine were frozen as described in chapter 2.

An ATP Bioluminescent Assay Kit was used to quantify ATP expression in sampled urine (Sigma-Aldrich). Standard solutions were prepared using the supplied ATP standard ($2x10^{-6}$ M ATP) and sterile 1% PBS as a diluent. Five serial dilutions of the ATP standard were prepared, producing solutions between $2x10^{-7}$ and $2x10^{-12}$ M ATP; the stock ATP standard and PBS used as $2x10^{-6}$ M and zero standards respectively.

Urine samples underwent only once freeze thaw cycle. Urine samples were thawed to room temperature and mixed using a vortex mixer (Scientific Industries, New York,

USA). The thawed samples were buffered to approximately pH 7.8 to optimise luciferase enzymic action. Sixteen wells in a 96 well microplate were each filled with 50 µl of the eight ATP standard solutions in duplicate; the remaining wells were filled with 50 µl of each urine test sample. The luciferin/luciferase reagent was reconstituted in the supplied diluent immediately prior to use. Fifty microlitres of the solution was added to each of the wells and bioluminescence measurements made immediately using a Synergy 3 luminometer (Biotek, Potton, UK) set at 570 nm.

3.7.1 Blinding

Microscopy and ATP analysis were performed with blinding to patient details and symptoms of participants. Samples presented for analysis were identified only by a randomly generated four-digit study number.

3.7.2 Evaluation of the effect of time on urinary ATP

Urine samples used to assess stability of ATP over time were stored at room temperature (23°C). Aliquots were taken at 0 hours (immediately), 12 hours, 24 hours, 48 hours, 168 hours and frozen at -80°C.

3.7.3 Evaluation of the effect of storage temperature on urinary ATP

The literature tends to recommend storage at -80°C; however, this is not convenient for many clinical services. The effects of freezing at -80°C and -20°C were therefore studied. 2ml aliquots of fresh urine were taken from each participant. One aliquot was frozen immediately after microscopy and stored at -80°C and another at -20°C.

3.7.4 Evaluation of the use of boric acid preservative on urinary ATP

The effects of boric acid preservation on decay of urinary ATP were studied. 10 mls of urine was introduced into pre-prepared boric acid tubes (Becton Dickinson Vacutainer® C&S Preservative Urine Tubes for Culture and Sensitivity) and stored at room temperature. Aliquots were taken at 0 hours (immediately), 12 hours, 24 hours, 48 hours, 168 hours and frozen at -80°C.

3.7.5 Evaluation of the effect of centrifugation on urinary ATP

Urinary ATP may originate from several sources including bacteria, urothelial cells and white cells. Therefore, I sought to discover whether centrifuging the urine altered the assay result, either by removing cells from the supernatant, or by lysing cells in the process. An aliquot of urine was spun at 620 g for 5 minutes and the supernatant was frozen at -80°C.

All of the urine aliquots were frozen immediately at -80°C. These samples were processed for urinary ATP between eight to twelve weeks after collection and storage. The frozen urine aliquots were thawed to room temperature (23°C) using a water bath and then immediately analysed using the standard luciferin-luciferase assay and protocol, as described earlier.

3.8 Statistical analysis

Multivariate linear regression analyses were used to scrutinise the $\log_{10}$ ATP as the response variable using two models. In the first, the explanatory variables were gender (0=female, 1=male); age; average 24-hour frequency; average 24-hour incontinence; number of stress incontinence symptoms, pain symptoms, voiding symptoms and OAB

symptoms; the presence or absence of any pyuria (0=none, 1= any pyuria); and the MSU culture result (0=negative, 1=positive). The second model looked more closely at the effect of the degree of pyuria. Pyuria was grouped as zero pyuria, pyuria 1-9 or pyuria ≥10, subgroups that are currently used by most clinicians, and these were referenced to control samples. The sample had 83% power to detect a .04 increment in R^2, if ten predictor variables were included in the regression model with alpha = 0.05. In the laboratory experimental series, paired data was collected and hence a paired t-test was used to analyse the difference in $\log_{10}$ ATP between paired samples stored at -20°C and -80°C; paired samples stored with and without boric acid; and paired samples centrifuged or not centrifuged. The diagnostic potential of urinary $\log_{10}$ ATP was assessed by ROC plots using positive MSU at 10^5 cfu ml^{-1} of a pure isolate of a known urinary pathogen; pyuria ≥ 10 wbc μl^{-1} and pyuria > 0 wbc μl^{-1}.

3.9 Results

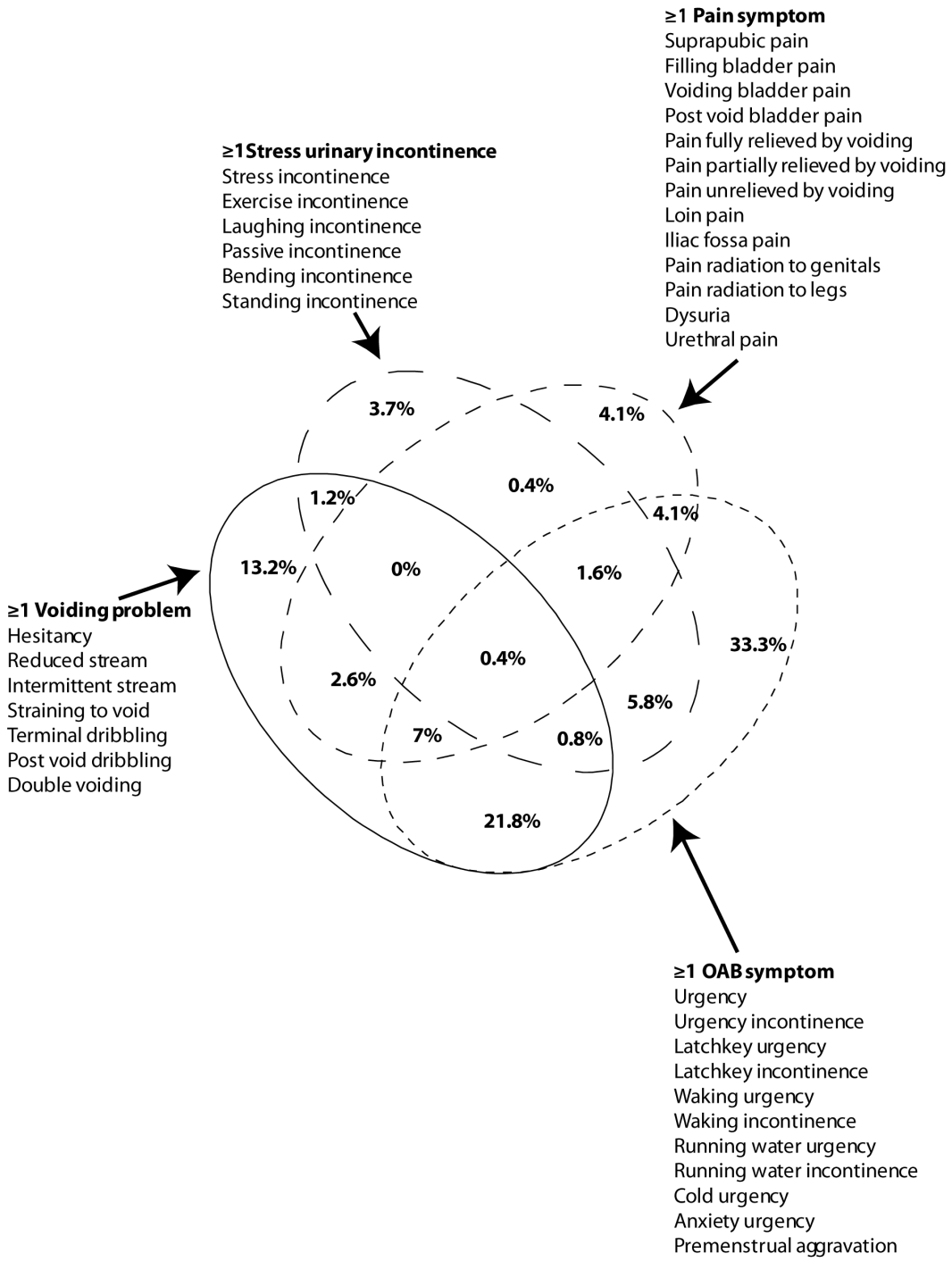
Urine samples from 75 healthy controls and 340 patients presenting with LUTS were collected. The patient cohort was grouped in the first model; with pyuria (≥1 wbc μl^{-1}) or without pyuria (0 wbc μl^{-1}). The second model used categorical scaling to compare pyuria 1-9 wbc μl^{-1} (n = 120) and pyuria ≥10 wbc μl^{-1} (n = 120) with a baseline factor of zero pyuria (0 wbc μl^{-1}). The demographic data can be seen in Table 3.1. The control group consisted of 49 females and 26 males, with mean age 38.2yrs (95% CI 34.5 - 41.8). Within the LUTS group there were 314 females and 26 males, with a mean age of 58.6yrs (95% CI 56.8 - 60.4) (Table 1). Of those with LUTS, 33.3% had only OAB symptoms, 4.1% had pain alone, 3.7% had only stress incontinence and 13.2% had only voiding dysfunction. Patients had a median of 3.5 LUTS (quartile range 1 to 6). The overlap of symptoms is illustrated in Figure 3.1.

Table 3.1 - Demographic data

	Controls					**LUTS patients**			
Gender male	N = 26					N = 26			
Gender female	N = 49					N = 314			
No Pyuria	N = 58 (female = 35, male = 23)					N = 100 (female = 92, male = 8)			
Any Pyuria	N = 17 (female = 14, male = 3)					N = 240 (female = 222, male= 18)			
	Mean		**Std Deviation (sd)**			**Mean**		**Std Deviation (sd)**	
Age (years)	38.2		15.8			58.6		16.6	
	Mean	**Median**	**sd**	**Quartile range**		**Mean**	**Median**	**sd**	**Quartile range**
24 hour frequency	6.2	6.2	6.0 to 7.0	6.0 to 7.0		9.2	8.0	8.0 to 10.0	6.0 to 11.0
24 hour incontinence	0	0	0.0 to 0.0	0.0 to 0.0		0.8	0.5	0.5 to 1.2	0.0 to 1.0
Number of urgency symptoms	0	0	0.0 to 0.0	0.0 to 0.0		2.8	2.0	2.0 to 3.6	0.0 to 4.0
Number of pain symptoms	0	0	0.0 to 0.0	0.0 to 0.0		0.4	0.0	0.2 to 0.6	0.0 to 0.0
Number of stress inc symptoms	0	0	0.0 to 0.0	0.0 to 0.0		0.3	0.0	0.08 to 0.5	0.0 to 0.0
Number of voiding symptoms	0	0	0.0 to 0.0	0.0 to 0.0		1.4	0.0	1.0 to 1.8	0.0 to 2.0
Number of LUTS	0	0	0.0 to 0.0	0.0 to 0.0		5.0	3.5	4.0 to 6.0	1.0 to 6.0

Figure 3.1- Venn diagram of symptom analysis

A four-way Venn diagram illustrating the overlap of symptom amongst the patients studied. The ellipses circumscribe patients who had one or more symptoms in the particular subset. The diagram is not scaled to the size of sets.



Log_{10} transformation of ATP changed the skewness from 4.2 to -0.3 and kurtosis from 27.5 to 1.1. The results of the two regression analyses are shown in Table 3.2. It can be seen that female gender was associated with higher predictions of the log_{10} ATP but given the small number of males, the limited discriminatory power precludes extrapolation. Voiding symptoms were also predictive of higher log_{10} ATP., Voiding symptoms in both sexes have been reported to be associated with inflammatory disease of the lower urinary tract (130). A positive culture result predicted lower log_{10} ATP. The first regression model shows that the presence of any pyuria was not predictive of higher log_{10} ATP. The second regression model demonstrates that a pyuria ≥10 wbc μl^{-1} was predictive of a higher log_{10} ATP; however, lower levels of pyuria 1-9 wbc μl^{-1} were not predictive. These data demonstrate that urinary ATP is elevated in association with inflammation but that urinary ATP lacks the discriminating properties at lower levels of pyuria, which would be essential for a useful clinical surrogate marker. This is illustrated by the ROC analysis which showed an area under the curve of 0.6 for a positive culture; 0.5 for pyuria > 0 wbc μl^{-1} and 0.6 for pyuria ≥ 10 wbc μl^{-1} (Figure 3.2). These data imply that there is no useful diagnostic role for this assay. The regression analyses showed that, age, average 24-hour frequency, average 24-hour incontinence, the number of urgency, stress incontinence, and pain symptoms provided no substantial explanation of the variance of urinary log_{10} ATP.

Table 3.2 - Output from regression analysis

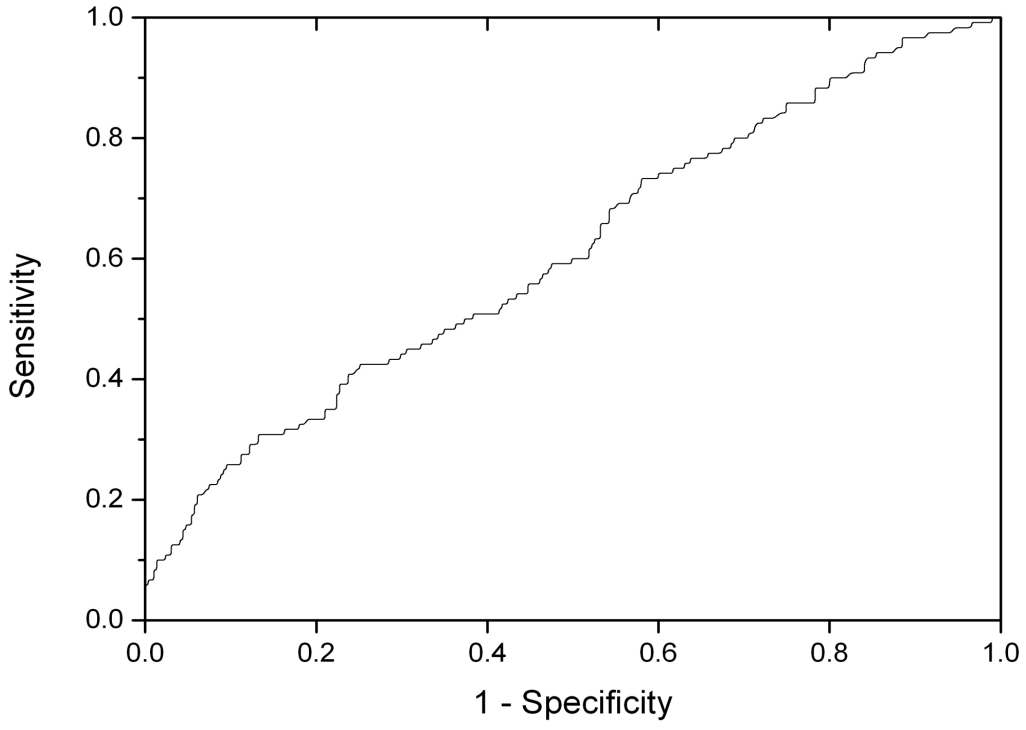
Model 1 Pyuria described by dichotomy				
			95% Confidence Interval for B	
	B Coefficient	p	Lower Bound	Upper Bound
(Constant)	-8.026	.000	-8.400	-7.652
Age	.004	.197	-.002	.009
Gender 0 = female, 1 = male	**-.537**	**.001**	**-.865**	**-.209**
MSU 0=negative 1=positive	**-.346**	**.004**	**-.582**	**-.110**
Average 24-hour frequency	-.004	.743	-.026	.019
Average 24-hour incontinence	-.001	.990	-.092	.090
Number of stress incontinence symptoms	-.065	.179	-.161	.030
Number of voiding symptoms	**.124**	**.000**	**.061**	**.187**
Number of pain symptoms	.082	.382	-.103	.267
Number of urgency symptoms	-.030	.163	-.072	.012
Pyuria 0= none 1= any	.157	.107	-.034	.349

Model 2 Pyuria described by ordinal scale				
			95% Confidence Interval for B	
	B Coefficient	p	Lower Bound	Upper Bound
(Constant)	-8.058	.000	-8.431	-7.685
Age	.002	.404	-.003	.008
Gender 0 = female, 1 = male	**-.522**	**.002**	**-.845**	**-.199**
MSU 0=negative 1=positive	**-.406**	**.001**	**-.642**	**-.171**
Average 24-hour frequency	-.002	.878	-.024	.021
Average 24-hour incontinence	-.024	.607	-.114	.067

Number of stress incontinence symptoms	-.060	.210	-.154	.034
Number of voiding symptoms	**.106**	**.001**	**.043**	**.169**
Number of pain symptoms	.085	.356	-.096	.266
Number of urgency symptoms	-.024	.257	-.066	.018
Pyuria1 to 9 wbc μl^{-1}	.207	.059	-.008	.422
Pyuria ≥ 10 wbc μl^{-1}	**.432**	**.000**	**.203**	**.662**

Dependent variable is urinary $\log_{10}$ ATP concentration

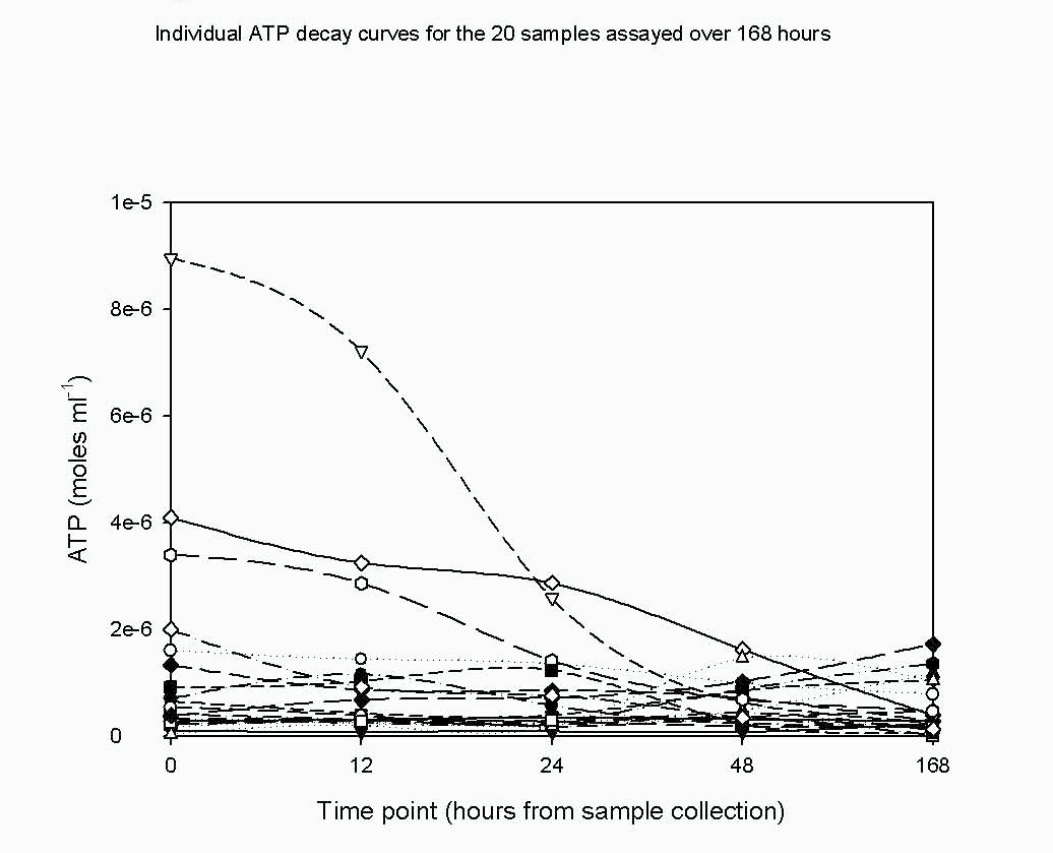
Figure 3.2 – Receiver-operator characteristics (ROC) curve for urinary ATP for the diagnosis of UTI. The ROC analysis shows an area under the curve of 0.6 for pyuria ≥ 10 wbc μl^{-1}. These data imply that there is no useful diagnostic role for this assay.



Urinary ATP decay over time: A subgroup of 20, randomly selected patient samples was used to plot the urinary ATP concentration in samples at differing time points after collection; 0 hours, 12 hours, 24 hours, 48 hours, 168 hours. Aliquots were taken at each point and frozen at -80ºC and stored for assays in batches. The ATP concentration fell with time with the rate of decline dependent on the initial concentration, as illustrated in Figure 3.3, where the time course of each sample is plotted.

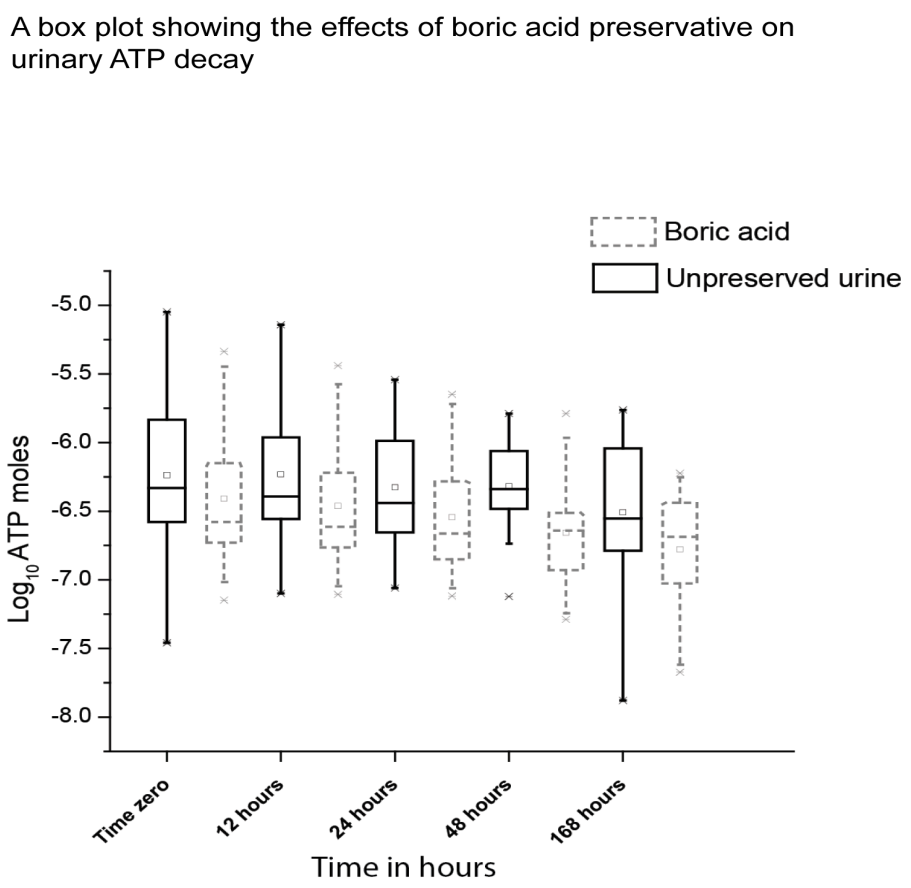
Figure 3.3 - The ATP decay curves for a subset of 20 urine samples

The rate of decay is substrate concentration dependent; such that a sample with a higher ATP concentration shows a more marked decay. It is therefore important to assay or freeze immediately after collection of the urine specimen to preserve the ATP signal.



Urinary ATP and effect of storage with boric acid preservative: Figure 3.4 shows box plots of the log_{10} ATP concentration at each time point comparing the effect of boric acid. An analysis of 20 paired samples at 24-hours demonstrated the significance of this difference: mean log_{10} ATP (moles) in samples stored without boric acid was -6.3 log_{10} moles and in samples stored with boric acid was -6.5 log_{10} moles (95% CI difference 0.15 to 0.29, t=6.2, p<.001). These data show that boric acid caused loss of ATP.

Figure 3.4 – A box plot showing the effects of boric acid preservative on urinary ATP decay. The box plot shows a subset of 20 paired samples to test the effect of boric acid as a urinary preservative on the decay of urinary ATP. Urinary ATP was measured at 12 hour intervals in urine samples with and without boric acid.



Urinary ATP and effect of storage temperature: Comparison of 30 paired samples of urine stored at -20°C and -80°C showed no significant difference in ATP concentration: mean $\log_{10}$ ATP (moles) in samples stored at -20°C was -6.7 $\log_{10}$ moles and in samples stored at -80°C was -6.8 $\log_{10}$ moles (95% CI difference -0.09 to +0.01 t=-1.7, p=.1). Thus storage at -20°C for 8 weeks would seem reasonable.

Urinary ATP and effect of centrifugation: Comparison of 30 paired samples of urine unspun and spun showed that the supernatant urine had a slightly lower level of ATP: mean $\log_{10}$ ATP (moles ml^{-1}) in uncentrifuged samples = -6.9 $\log_{10}$ moles and in the supernatant after centrifuge mean = -6.8 $\log_{10}$ moles (95% CI difference 0.03 to 0.1, t = 3.5, p=.002). Whilst statistically different, this is a very small difference and of little clinical significance.

3.10 Discussion

ATP has been proposed as a potential clinical marker for acute and chronic urinary tract disease (33, 131). To avoid premature use, when assessing the diagnostic potential of test, it is important to assess first whether the measure explains the symptoms and other manifestations of the disease of interest (132). The data published here demonstrate that urinary ATP shows little concordance with the clinical consequences of urinary infection in chronic LUTS, and therefore does not show promise for future development of a diagnostic test for this particular disease. This observation is confirmed in the ROC curves that explore sensitivity and specificity properties.

These results demonstrate that patients with LUTS and any pyuria do not manifest a significantly raised urine ATP concentration. It is only when there is a high pyuria of greater than 10 wbc μl^{-1}, which is a currently used marker of urinary infection, that

there is a significantly raised urinary ATP. However, this signal was not a discriminating marker for low levels of lower urinary tract inflammation (pyuria 1-9 wbc μl^{-1}) as shown by our second regression model, and it is in these clinical circumstances that there is greatest need for novel clinical surrogate markers. It has been shown that low-level pyuria (133), voiding symptoms (130), overactive bladder symptoms (55) and pain symptoms (134) are all correlated with urinary infection. Only voiding symptoms explained a small amount of the variance in ATP. These findings are therefore discouraging.

Counter-intuitively, urinary ATP was lower, given a positive urine culture. This unexpected result may reflect the fact that there were so few positive cultures (16.9%). Increased microbial dephosphorylation of ATP to adenosine, which we did not assay, may also be a contributing factor. In addition, the urinary ATP reflects the urinary microbial biomass directly post void and there may be significant variation in the urinary biomass at the time of assay secondary to transportation and processing delay.

There was an unbalanced sample size with fewer control participants with a lower average age, and this reflects the common difficulty of finding older subjects without any urinary tract symptoms. The controls in this experiment were all asymptomatic. I did control for these differences in the analysis but would nevertheless wish to avoid wide inferential generalisations. The essence of this study was to scrutinise the discriminating properties amongst patients. The recruitment field was also predominantly female which may explain the gender difference. The small numbers of males were nevertheless included in the analysis as the model was sufficiently powered for this variable, which required ≥20 for each independent variable. The sample was powerful enough to detect the influence of voiding symptoms, pyuria and culture,

despite the wide variance. Urinary ATP concentrations proved independent of age and the number and nature of LUTS symptoms, other than voiding symptoms. The association with voiding symptoms was not surprising because in patients with chronic LUTS, these have been reported to be correlated with pyuria in both genders, in clear contrast to OAB and pain symptoms (130).

These data suggest that urinary ATP could not pick out low levels of inflammation (pyuria 1-9 wbc μl^{-1}) when used alone, a situation where clinicians need most help. Current dipstick methods and direct urinary microscopy are able to identify pyuria when it is abundant (133), so urinary ATP would offer no additional advantage. There can be little doubt that ATP plays a significant role in the pathophysiology of urinary tract disease (135, 136) but it would seem that this does not translate into a useful role as a clinical test when used alone.

The laboratory experimental series showed that urine samples should be processed immediately or frozen and stored at below -20°C. There was a significant urinary ATP decay with time when samples were left at room temperature, with the rate being substrate dependent. We also found that storage of urine at -20°C is adequate and this therefore allows for wider use of standard freezers as opposed to the specialised -80°C devices. The centrifuge studies examined the effect of removing cellular material. The supernatant showed marginally lower levels of ATP. This could be attributed to increased ATPase activity from burst cells or it may have resulted from delayed freezing, allowing additional time for enzyme activity. Additionally, centrifugation results in the removal of biomass and hence this may explain the lower levels of urinary ATP. This marginal difference, though statistically significant, may not be clinically significant; nevertheless, centrifuging of urine prior to freezing or analysis is an additional labour

worth avoiding. Boric acid proved counterproductive and so should not be used as a preservative.

In summary these data discourage the idea that urinary ATP should be developed as a clinical surrogate test for UTI. This assay does not appear more effective than markers used in current clinical practice (45, 133). However, abundant urinary ATP is certainly evident in the presence of significant disease amongst patients with LUTS and these data do encourage continued interest in the pharmacology and pathophysiology of purinergic functions in the bladder.

In conclusion, urinary ATP does not improve on the use of microscopy as a surrogate marker of urinary tract infection and is therefore not a promising clinical diagnostic marker. There is need to explore other potential markers that can be used to screen LUTS patients for UTI, particularly applicable to those that have lower levels of pyuria, where significant disease may currently be overlooked (133). The relevance of measuring ATP to study the pathophysiology of the lower urinary tract is still very evident and in that context urine is a useful biological sample.

4 Chapter 4 – The cytokine response in OAB

4.1 Introduction

Contemporary published data show that microscopic pyuria, evaluated by inspections of a fresh, unspun, unstained specimen of urine using a haemocytometer remains our most reliable marker of microbial urinary infection. This requires a microscope and the accompanying skills to use and maintain the instrument. Thus, urinary inflammatory mediators offer a potential source of alternative, surrogate markers. Urinary tract infection stimulates the release of a variety of inflammatory mediators including platelet-derived growth factor (PDGF), tumour necrosis factor-α (TNF-α), IL6 and CXCL 8 (84). Urinary cytokines are elevated in patients with UTI and epithelial cells have been identified as early producers of cytokines in the murine UTI model (91) (92, 93) (94). Uroepithelial cell lines of bladder and kidney origin constitutively make IL-6 and respond with elevated IL-6 production to exogenous stimuli like bacteria or cytokines (91, 95, 96). Studies of patients with UTI have shown rapid increases in urine IL6 levels after the onset of infection or instillation of bacteria into the urinary tract but serum IL6 levels are only elevated in those with acute pyelonephritis (97-99). Through the release of cytokines, epithelial cells establish a network with local and distant host cells (95). Cell recruitment follows after the secretion of chemokines like IL-8. Studies have shown that IL-8 is present in epithelial cells of the healthy urinary tract and that IL-8 is secreted in response to infection. IL-8 then returns to non-detectable levels after treatment, and is absent from normal urine (100). There is very little information on inflammatory mediators and the role of infection in patients with overactive bladder symptoms. There are a few studies that have looked at inflammatory cytokines in OAB and have

found associations with NGF and MCP-1 (109-111, 137). Study methods have included ELISAs, whilst others have looked at microarrays and found inflammatory signals in patients with overactive bladder symptoms. These studies have not looked at the role of infection that may be missed by conventional methods of testing within these patients.

4.2 Hypothesis

Patients with OAB symptoms show a higher urinary inflammatory signal as compared to controls with higher levels of urinary Interleukin 6 (IL6), CXCL 8, Lactoferrin, Tamm-Horsfall Protein (THP), Monocyte Chemoatractant Protein-1 (MCP) and Nerve Growth Factor (NGF).

4.3 Study Design

A prospective controlled study was conducted to test the hypothesis and measure levels of urinary Interleukin 6, CXCL 8, Lactoferrin, Tamm-Horsfall Protein (THP), Monocyte Chemoatractant Protein-1 (MCP) and Nerve Growth Factor (NGF) in patients with OAB symptoms as compared to controls.

4.4 Ethical Approval

Studies included in this work had ethical approval by the National Research Ethics Service (NRES) - East Central Research Ethical Committee 1 (REC Reference number 11/H0721/7).

4.5 Inclusion and exclusion criteria

Subject Inclusion Criteria

1. Adults aged >=18 years
2. Able to complete a symptom questionnaire
3. Diagnosed with OAB with or without pyuria (≥ 1 wbc μl^{-1})
4. Post-menopausal women or women using adequate contraception

Subject Exclusion Criteria

1. Age less than 18 years
2. Inability to consent
3. Pregnant women or women planning to conceive
4. Patients with concurrent illnesses that in the opinion of the investigator are likely to compromise the validity of the data

The study groups were

1) Female adult patients with OAB, describing:

   a. Urgency, the sudden compelling desire to urinate, a sensation that is difficult to defer.
   b. Urinary frequency, voiding eight or more times in a 24-hour period.
   c. Nocturia, the need to wake one or more times per night to void.
   d. They may or may not experience urinary incontinence.
   e. With or without pyuria (≥1 wbc μL^{-1}) and/or a history of acute symptom exacerbations.

2) Control Group - Female adults without OAB and without other lower urinary tract symptoms.

4.6 Study Population

4.6.1 Recruitment

Patients with OAB symptoms and healthy volunteers were recruited as described in chapter 2.

4.6.2 Consent

Written consent was obtained from all participating individuals as described in chapter 2.

4.7 Safety considerations

There were no safety considerations for patients providing MSU samples only.

4.8 Data management

Study data were stored in the Department of Medicine, UCL Archway Campus, as per Good Clinical Practice (GCP) guidance. All samples and recoded data were identifiable by study numbers and participant initials. Any patient identifiable data was stored on only a secure NHS database, which was protected by encryption and daily backup.

4.9 Study Method

All participants provided clean catch MSU samples and immediate microscopy for pyuria and epithelial cells was performed. Aliquots of spun urine were frozen as described in chapter 2.

4.9.1 Blinding

Microscopy was performed with blinding to patient details and symptoms of participants. Samples presented for analysis were identified only by a randomly generated four-digit study number. Blinding was ensured during sample processing as samples were only identified by the four-digit study number.

4.10 Interleukin-6 (IL6)

4.10.1 Brief description of methods

A controlled, prospective cross-sectional study of IL6 expression in patients with OAB symptoms was undertaken. Female patients with symptoms of OAB and healthy volunteers were recruited as described in chapter 2 and written consent was obtained. All participants provided clean catch MSU samples and immediate microscopy for pyuria and epithelial cells was performed. Aliquots of spun urine were frozen at -80°C as described in chapter 2, for IL6 quantification using a high-sensitivity, enzyme-linked immunosorbent assay (ELISA). Patients and controls provided carefully collected MSU samples and aliquots of spun urine frozen as described in chapter 2.

The samples were thawed to room temperature and analysed in batches, and blinding to sample details and symptoms ensured. The Quantikine® High Sensitivity ELISA Human IL6 Immunoassay was used to quantify IL6 expression in urine (R&D Systems, Abingdon, UK), with a limit of detection of 0.09pg ml^{-1} with an inter- and intra-assay coefficient of variation of less than 10%. Frozen urine samples under went only one freeze thaw cycle ensuring stability of IL6. The urine samples were thawed to room temperature and mixed thoroughly using a vortex mixer (Scientific Industries, New York, USA). The assay included IL6 stock standard at a concentration of 10 pg ml^{-1}. Calibrator

diluent was used to produce serial dilutions ranging from 10 pg ml^{-1} to 0.156 pg ml^{-1}. Calibrator diluent was used as 0 pg ml^{-1} standard.

The supplied pre coated 96 well microplate was used to plate the IL6 standards and urine samples. 100 µl of the eight IL6 standards were plated in duplicate. 40 urine samples were plated in duplicate in the remaining 80 wells by adding 100 µl of each urine sample. The plate was then covered and incubated at room temperature. Binding was encouraged using an orbital microplate shaker (0.12" orbit) set at 500 ± 50 rpm for two hours.

After the 2 hour incubation the well contents were remove and the wells washed six times with wash solution. Each wash was carefully pipetted without disruption to the base of the well. In a second incubation, 200µl of IL6 conjugate was then added to each well, and the plate incubated for a further two hours. The well contents were washed as described earlier. The plate was then incubated on the bench top, after addition of 50µl of substrate solution, at room temperature for one hour. Following this was the addition of 50 µl of amplifier solution without any washing and a colour change noted. The plate was incubated on the bench top at room temperature for 30 minutes. In the final step 50µl of stop solution was added followed by analysis within 30 minutes. Interleukin-6 concentration was determined using an Opsys MR fluorescence microplate reader (DYNEX Technologies, Worthing, UK), set at 490 nm. The reader produced standard curves, which were used to calculate concentrates for each well. The minimum detectable dose (MDD) of the ELISA kit according to the manufacturers ranges from 0.016-0.110 pg ml^{-1}, with an average MDD of 0.039 pg ml^{-1}. All samples were analysed in duplicate to test the inter-assay precision and the mean value was

taken. The intra-assay precision for urine assays suggested by the company was 5.5-9.8% and the inter-assay precision for this experiment was 5.5-11.2%.

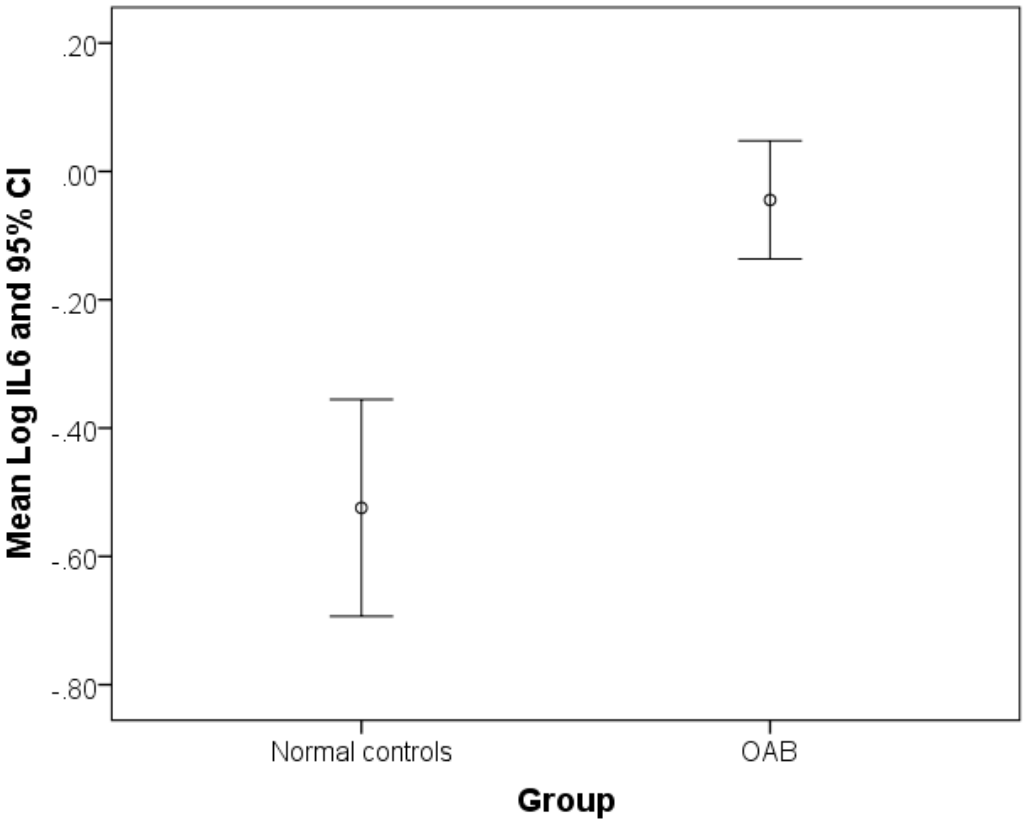
4.10.2 Statistical Analysis

The power to test the null hypothesis that the population means were equal was calculated. The criterion for statistical significance (alpha) was set at 0.05. The test was 2-tailed. It was found that a sample size comprising 20 controls and 20 patients had a 80% power to detect 0.4 pg ml^{-1} difference in IL6 between patients and controls, given a population standard deviation of 0.4 pg ml^{-1}. The data were checked for normality using a Q-Q plot and found to be satisfactory, hence parametric methods were used for analysis. Statistical analysis of the data was conducted using ANOVA at the 95% level of confidence.

4.10.3 Results

One hundred and eighty two patients (F=179; M=33; mean age (years)=60.0; sd=17.7) and 30 control subjects (F=11; M=19; mean age (years)=25.1; sd=9.3) were included in the analysis. There was no significant difference in urinary IL6 when compared with gender (p=.29, 95% CI -0.11 to 0.36). Urinary IL6 expression was significantly greater in patients with OAB symptoms compared to asymptomatic controls (ANOVA F=16; p<.001, 95% CI of difference -0.7 to -0.24) as seen in figure 4.1.

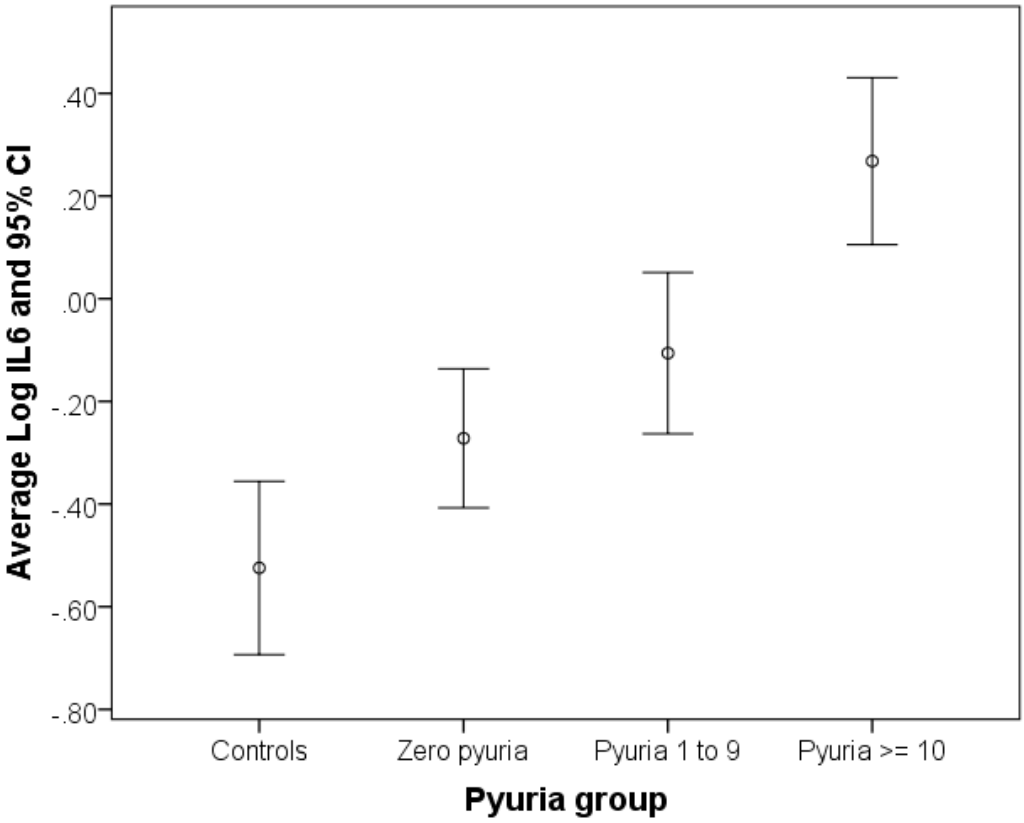
Figure 4.1 – A graph to show the mean Log urinary IL6 and 95% confidence interval in patients with OAB and controls



Amongst patients, pyuria was associated with higher levels of IL6, although patients without pyuria still demonstrated greater IL6 expression than normal controls (ANOVA $F=27.2$, $p<.001$), shown in figure 4.2. However Bonferroni post hoc analysis showed a significant between group difference when comparing controls with OAB patents with pyuria 1- 9 or greater than 10. There was no significant difference when comparing OAB patients with zero pyuria and pyuria 1-9. OAB patients with a pyuria of greater than 10 differed from all groups.

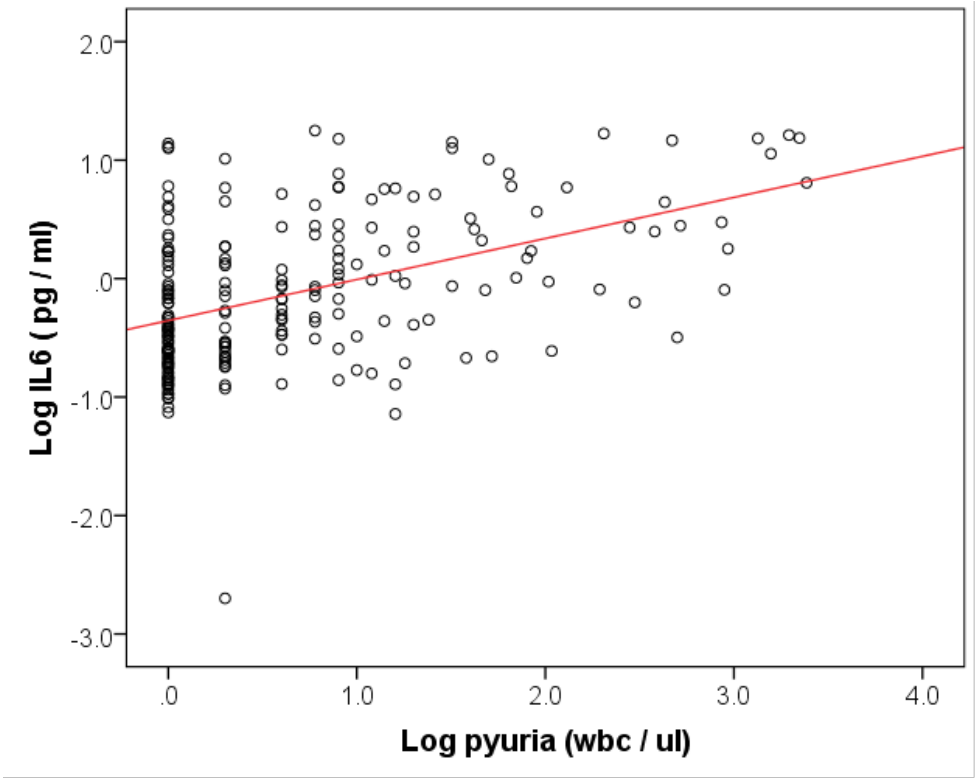
Despite differences in the demographic characteristics of the study groups, these findings support preliminary data demonstrating cytological evidence of urothelial inflammation in patients with OAB symptoms.

Figure 4.2 – A graph to show the mean log urinary IL6 and 95% confidence interval in patients with OAB no pyuria, OAB with pyuria and controls.



We found a correlation between Log IL6 and Log pyuria (R = 0.46, F=59, p<.001). A linear regression model was fitted to the data with log Lactoferrin as the dependant variable with R calculated at 0.46 (Figure 4.3)

Figure 4.3 – A graph to show the correlation between Log IL6 and Log pyuria.



4.10.4 Discussion

There are significant data to suggest that a variety of bladder epithelial cell derived mediators are involved in the elevated neutrophil response in the bladder after acute bacterial infection (88, 96). IL6 has shown a prominent role in patients with acute UTI though there is some debate about the pathways involved (138). It is thought that in adaptation to uropathogenic bacterial suppression of an inflammatory response, there may be multiple pathways by which bladder epithelial cells are able to trigger response with elevated IL6 (95). Identifying a cytokine response in patients with OAB supports the premise that some patients with OAB may have an inflammatory aetiology to their condition. In addition, in patients with OAB and pyuria we have found an increased level of urinary IL6. The association between IL6 and urine infection in the literature also leads us to postulate on an infective aetiology in these patients. In addition, there is growing evidence in the literature that the magnitude of the cytokine response and the

type of cytokine found in the urine is influenced by the virulence of the pathogen. IL6 has been particularly associated with fimbriae (96, 139). The findings in this study support the hypothesis that OAB symptoms may be associated with a urothelial inflammatory response, which may be secondary to urine infection.

4.11 Lactoferrin

4.11.1 Brief description of methods

A controlled, prospective cross-sectional study of urinary Lactoferrin in patients with OAB symptoms was undertaken. Female patients with symptoms of OAB and healthy volunteers were recruited as described in chapter 2 and written consent was obtained. All participants provided clean catch MSU samples and immediate microscopy for pyuria and epithelial cells was performed. Aliquots of spun urine were frozen at -80°C as described in chapter 2, for urinary Lactoferrin quantification using a high sensitivity sandwich enzyme-linked immunosorbent assay (ELISA).

Urine samples underwent only one freeze thaw cycle to ensure stability of Lactoferrin. Urine samples were thawed to room temperature and mixed using a vortex mixer (Scientific Industries, New York, USA). Samples were only identifiable by study numbers to ensure blinding to sample details and group. The Human Lactoferrin ELISA immunoassay was used to quantify urinary Lactoferrin (ICL, Portland, USA), with a range of detection of 3.125ng ml^{-1} -100ng ml^{-1}, with a sensitivity average of 0.725ng ml^{-1}. Lactoferrin standard solutions were prepared using 100 ng ml^{-1} stock lactoferrin standard and six serial dilutions using calibrator diluent. This produced standard solutions between 100ng ml^{-1} and 3.125ng ml^{-1}, and calibrator diluent was used as zero standard.

Fourteen wells in the supplied microplate were filled 100 µl of the seven Lactoferrin standard solutions in duplicate, with the remaining wells accommodating 100 µl of each urine test sample. The plate was incubated on the bench top at room temperature for 30 minutes to allow binding. Following incubation well contents were aspirated and the wells carefully filled with wash solution. This was then carefully aspirated and the process repeated 3 times.

After washing, 100µl of Enzyme Antibody Conjugate was added to each well, and the plate incubated for a further 30 minutes. The well contents were again emptied and washed four times. One hundred microlitres of the supplied substrate solution were then added to each well, and the plate incubated on in a dark room at room temperature for 10 minutes. One hundred microlitres of the supplied stop solution were then added to each of the wells and analysis undertaken within 30 minutes. Lactoferrin concentration was determined using an Opsys MR fluorescence microplate reader (DYNEX Technologies, Worthing, UK), set at 450 nm. The reader produced standard curves, fit to a four-parameter logistics curve, which were used to calculate concentrates for each well. All samples were analysed in duplicate to test the inter-assay precision and the mean value was taken.

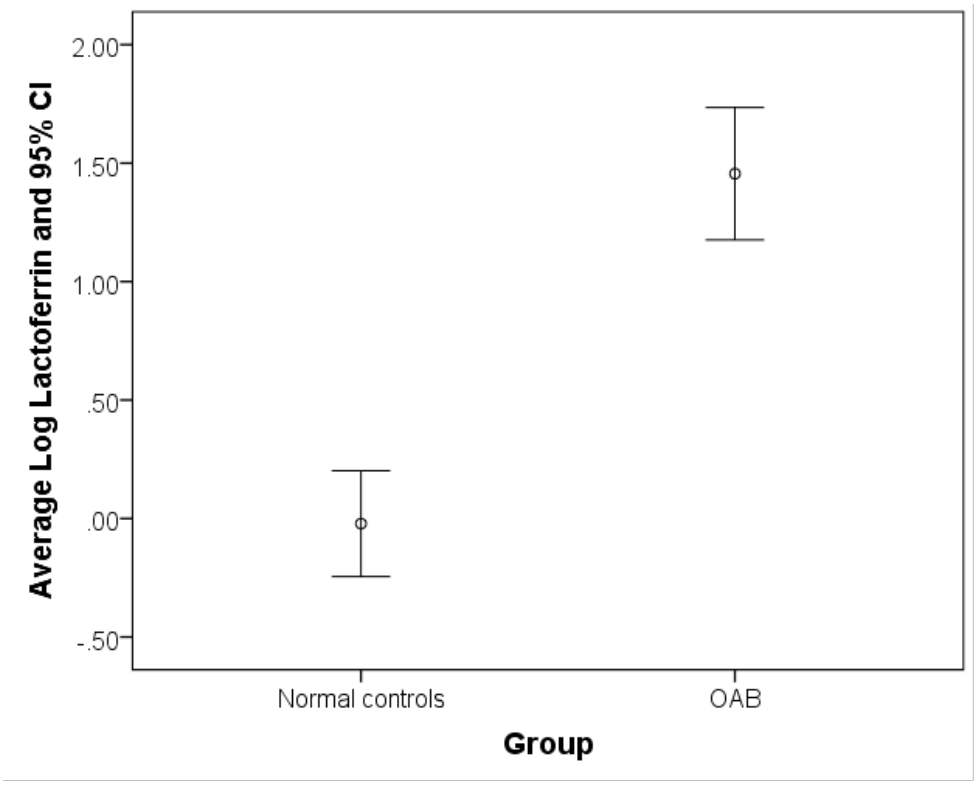
4.11.2 Statistical Analysis

The data were checked for normality using a Q-Q plot and found to be satisfactory, hence parametric methods were used for analysis. Statistical analysis of the data was conducted using ANOVA at the 95% level of confidence. Correlation between Lactoferrin and pyuria was explored using a regression analysis.

4.11.3 Results

Sixty five patients (F=59; M=6; mean age (years)=62.3; sd=16.9) and 14 control subjects (F=9; M=5; mean age (years)=53.6; sd=16.9) were included in the analysis. Urinary Lactoferrin was significantly greater in patients with OAB compared to asymptomatic controls (ANOVA F=23.2; p<.001, (95% CI -2.1 to -0.87) as seen in figure 4.4.

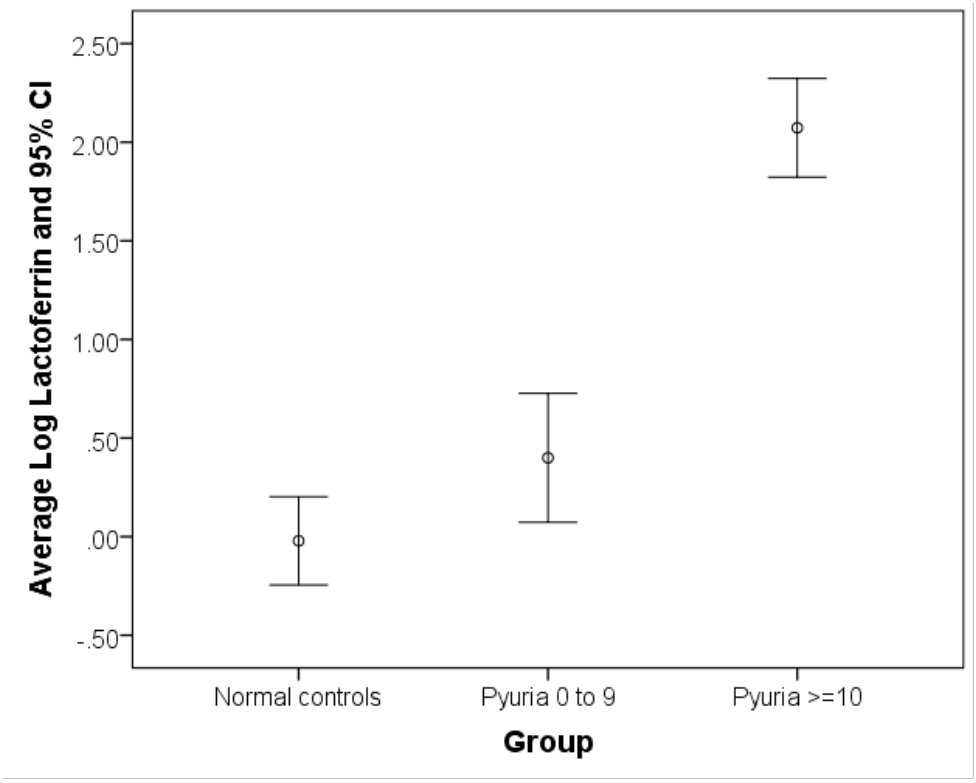
Figure 4.4 - A graph to show the mean Log urinary Lactoferrin and 95% confidence interval in patients with OAB and controls



Amongst patients, only 3 patients had zero pyuria, 21 had pyuria 1 – 9 and 41 had pyuria of 10 or more. Therefore, we grouped patients into those with pyuria of 0-9 and those with pyuria of ≥10. Patients with pyuria 0-9 and those with pyuria ≥10 demonstrated greater urinary Lactoferrin when compared with controls (ANOVA F=62.8; p<.001), shown in figure 4.5. However post hoc analysis showed a significant between group difference when comparing controls with OAB patents with pyuria ≥10

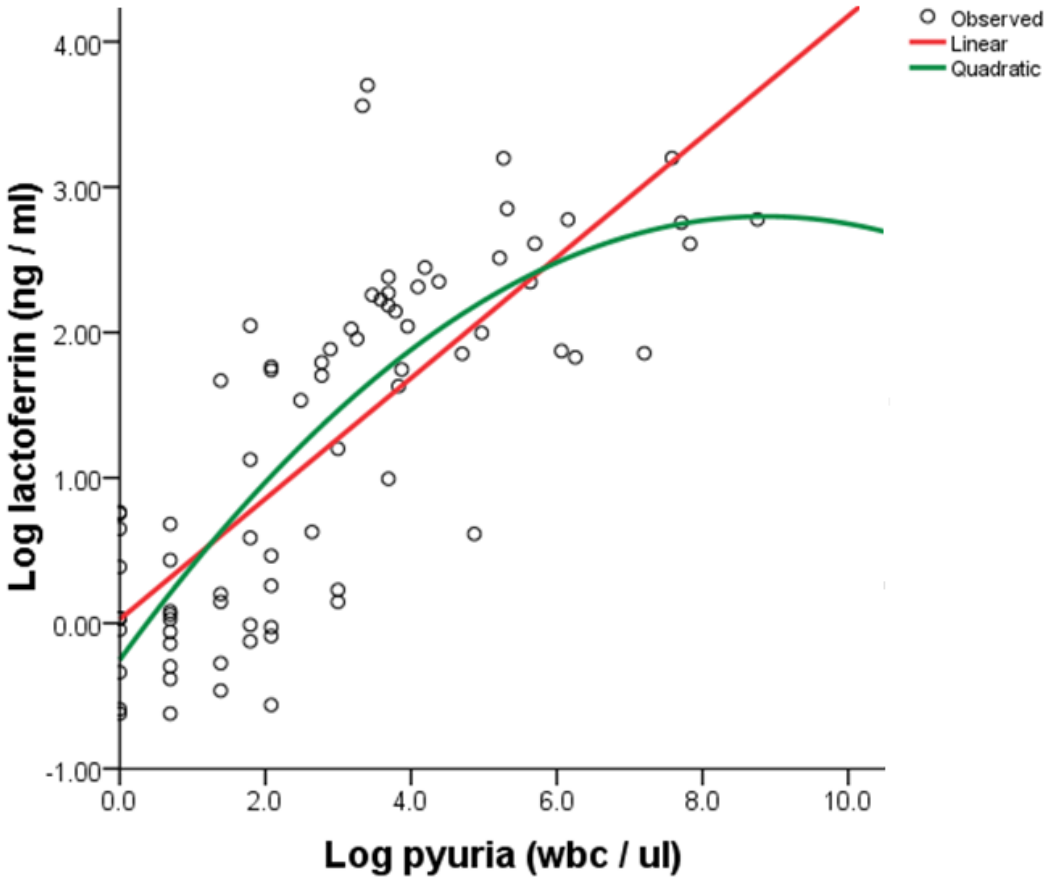
but no significant difference when comparing controls and OAB patients with pyuria 0-9.

Figure 4.5 – A graph to show the mean Log urinary Lactoferrin and 95% confidence interval in patients with OAB pyuria 0-9 , OAB pyuria greater than or equal to 10 and controls



We found a correlation between Log Lactoferrin and Log pyuria (R = 0.8, F=112, p<.001). A quadratic regression model was fitted to the data with log Lactoferrin as the dependant variable with R calculated at 0.8 (Figure 4.6)

Figure 4.6 – A graph to show the correlation between Log Lactoferrin and Log pyuria



4.11.4 Discussion

These data demonstrated that urinary Lactoferrin levels discriminate successfully between patients with OAB and pyuria. There is good evidence that the Lactoferrin reflects the pyuria status quantitatively and so may warrant further exploration in relation to other markers of inflammation and infection. This is encouraging data from this small pilot study however now would need further robust evaluation with a range of other surrogate markers of disease and quantitative measure of symptoms.

4.12 CXCL 8

4.12.1 Brief description of methods

A controlled, prospective cross-sectional study of urinary CXCL 8 in patients with OAB symptoms was undertaken. Female patients with symptoms of OAB and healthy volunteers were recruited as described in chapter 2 and written consent was obtained. All participants provided clean catch MSU samples and immediate microscopy for pyuria and epithelial cells was performed. Aliquots of spun urine were frozen at -80°C as described in chapter 2, for urinary CXCL 8 quantification using a high sensitivity enzyme-linked immunosorbent assay (ELISA).

Urine samples underwent only one freeze thaw cycle to ensure stability of CXCL 8. Urine samples were thawed to room temperature and mixed using a vortex mixer (Scientific Industries, New York, USA). Samples were only identifiable by study numbers to ensure blinding to sample details and group. The Human CXCL 8 ELISA immunoassay was used to quantify urinary CXCL 8 (R and D systems), with a mean minimal detection range of with a range of detection of 3.5pg ml^{-1}. The assay included CXCL8 stock standard at a concentration of 2000 pg ml^{-1}. Calibrator diluent was used to produce serial dilutions ranging from 2000 pg ml^{-1} to 31.2 pg ml^{-1}. Calibrator diluent was used as 0 pg ml^{-1} standard. All wells were filled with 100 µl of assay diluent. Sixteen wells in the supplied microplate were filled with 50 µl of the CXCL 8 standard solutions in duplicate, with the remaining wells accommodating 50 µl of each urine test sample. The plate was then incubated at room temperature for 2 hours.

After the 2 hour incubation the well contents were remove and the wells washed six times with wash solution. Each wash was carefully pipetted without disruption to the

base of the well. In a second incubation, 100µl of CXCL 8 conjugate was then added to each well, and the plate incubated for a further 1 hour. The well contents were washed as described earlier. The plate was then incubated with protection from light, after addition of 200µl of substrate solution, at room temperature for 30 minutes. In the final step 50µl of stop solution was added followed by analysis within 30 minutes. CXCL 8 concentration was determined using an Opsys MR fluorescence microplate reader (DYNEX Technologies, Worthing, UK), set at 450 nm. The reader produced standard curves, fit to a four-parameter logistics curve, which was used to calculate concentrates for each well. All samples were analysed in duplicate to test the inter-assay precision and the mean value was taken.

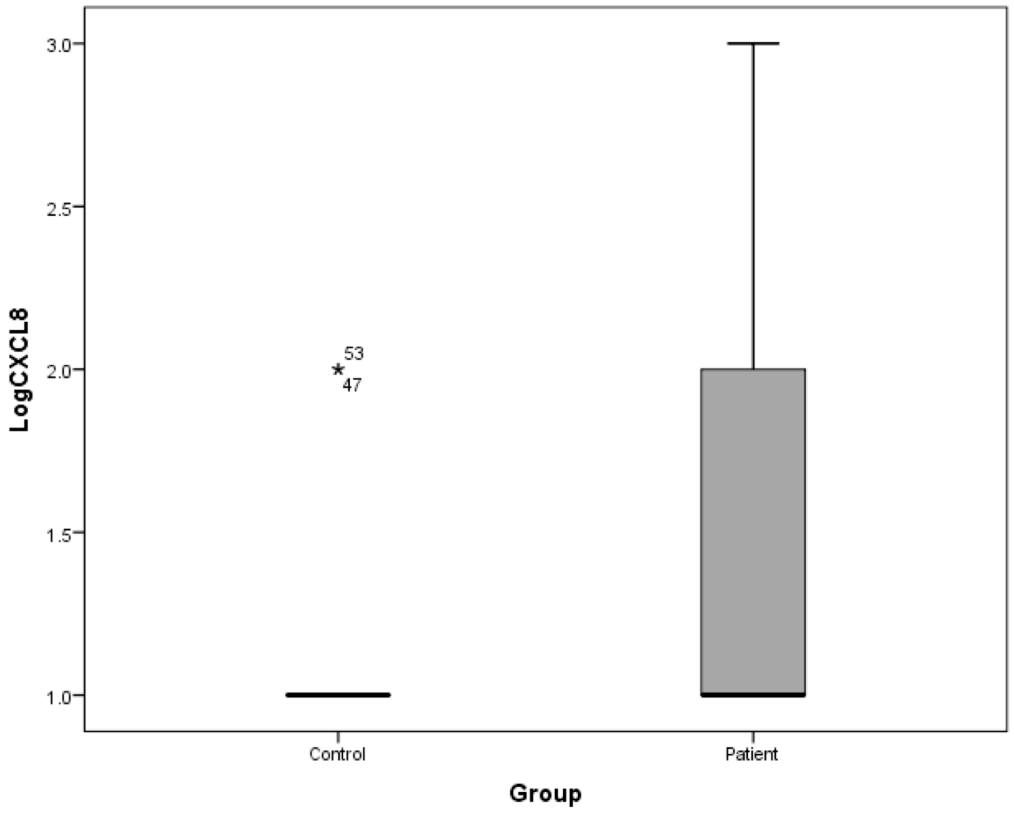
4.12.2 Statistical Analysis

The data was assessed for normality using a Q-Q plot and found not to be normally distributed, hence non-parametric methods were used for analysis. Statistical analysis of the data was conducted using Mann Whitney U test and Kruskall-Wallis to assess for a between group difference.

4.12.3 Results

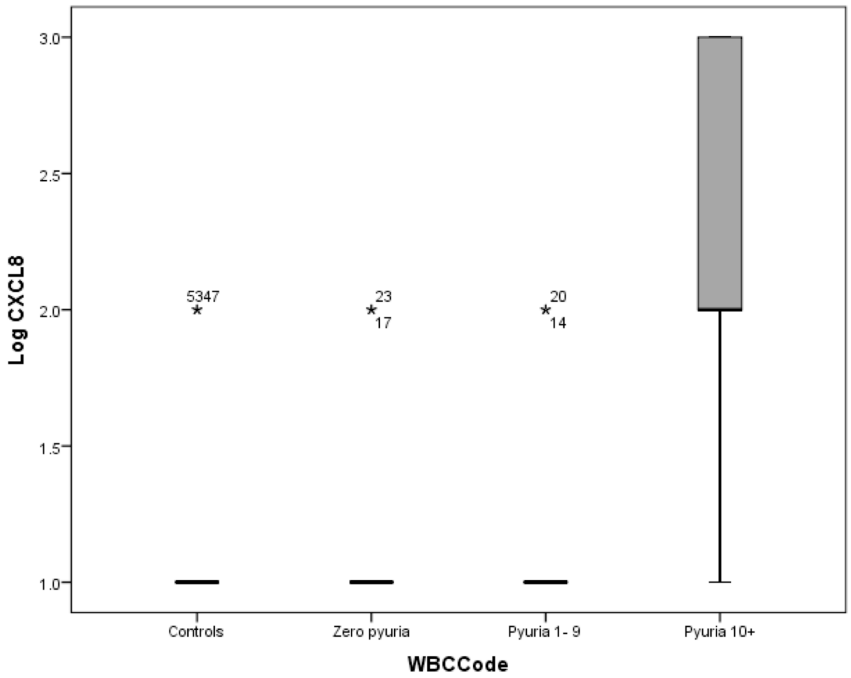
Forty four patients (F=41; M=3; mean age (years)=53.1; sd=18.0) and 12 control subjects (F=6; M=6; mean age (years)=40.9; sd=16.2) were included in the analysis. Urinary CXCL 8 was not significantly different when comparing patients with OAB compared to asymptomatic controls (Mann Whitney U Z=-1.63; p=.103), as seen in figure 4.7.

Figure 4.7 – A box plot showing Log CXCL 8 in controls and patients with OAB



When comparing urinary CXCL 8 with pyuria there was significantly higher urinary CXCL 8 in patients with pyuria greater than 10 when compared with control, pyuria zero and pyuria 1-9 (Chi-square 24.4, df=3, p<.001) seen in figure 4.8.

Figure 4.8 – Box plot to show urinary CXCL 8 in controls and patients with increasing pyuria



4.12.4 Discussion

These preliminary data do not show a significant difference in urinary CXCL 8 when comparing normal healthy controls with patients with OAB. However it is difficult to form strong conclusions from a small sample. In addition, there was a significant difference in mean age between controls and patients and the sample size was small. On further analysis when comparing urinary CXCL 8 with pyuria status in this group there was significantly higher urinary CXCL 8 in patients with pyuria ≥10. However urinary CXCL 8 lacks the ability to differentiate between controls and patients with low levels of pyuria where current tests are needed. Therefore based on this small study urinary CXCL 8 does not show potential as a marker that would help distinguish between patients and controls.

4.13 Uromodulin / Tamm Horsfall protein (THP)

4.13.1 Brief description of methods

A controlled, prospective cross-sectional study of urinary uromodulin in patients with OAB symptoms was undertaken. Patients with symptoms of OAB and healthy volunteers were recruited as described in chapter 2 and written consent was obtained. All participants provided clean catch MSU samples and immediate microscopy for pyuria and epithelial cells was performed. Aliquots of spun urine were frozen at -80°C as described in chapter 2, for urinary Uromodulin quantification using a high sensitivity enzyme-linked immunosorbent assay (ELISA).

Urine samples underwent only one freeze thaw cycle to ensure stability of Uromodulin. Urine samples were thawed to room temperature and mixed using a vortex mixer (Scientific Industries, New York, USA). Samples were only identifiable by study numbers to ensure blinding to sample details and group. The Human Uromodulin glycoprotein ELISA immunoassay was used for the quantitative determination of Uromodulin Glycoprotein (Tamm-Horsfall Glycoprotein, THP) in urine samples (MDbioproducts). The sensitivity of the Uromodulin ELISA is described as typically less than 0.75 ng ml^{-1}. Sensitivity of the assay was defined by the manufacturer as the minimal detectable dose determined by adding two standard deviations of the mean optical density value for twenty replicates of the zero standard and calculating the corresponding concentration. Seven standard solutions were prepared using the supplied Uromodulin standard (2500 ng ml^{-1}) and assay diluent to produce a 2-fold dilution series, with 150 ng ml^{-1} standard serving as the highest standard and Assay Diluent serving as the zero (0 ng ml^{-1}). Human urine samples were diluted 1:200 and then serially diluted 1:2 in Assay Diluent.

Uromodulin Glycoprotein Microplate containing 12 x 8 strips coated with polyclonal antibody to Uromodulin Glycoprotein was used. Sixteen wells in the supplied microplate were filled with 50 µl of the Uromodulin standard solutions in duplicate, with the remaining wells accommodating 50 µl of each urine test sample. The plate was then incubated at 37 °C for 60 minutes. The well contents were then emptied and the wells washed six times using the supplied wash solution.

After washing, 50µl of Uromodulin Conjugate were added to each well, and the plate incubated for a further 60 minutes at 37 °C. The well contents were again emptied and washed six times. Fifty microlitres of the supplied diluted Streptavidin-HRP substrate solution were then added to each well, and the plate incubated for 30 minutes insuring protection from light. The well contents were then emptied and the wells washed six times using the supplied wash solution. Fifty microlitres of Substrate were added to each well and incubated for 10 minutes at 37 °C ensuring protection from light. Fifty microlitres of stop solution were then added to each of the wells and analysis undertaken within 30 minutes. Uromodulin concentration was determined using an Opsys MR fluoresence microplate reader (DYNEX Technologies, Worthing, UK), set at 450 nm. The reader produced standard curves, fit to a four-parameter logistics curve, which was used to calculate concentrates for each well. All samples were analysed in duplicate to test the inter-assay precision and the mean value was taken.

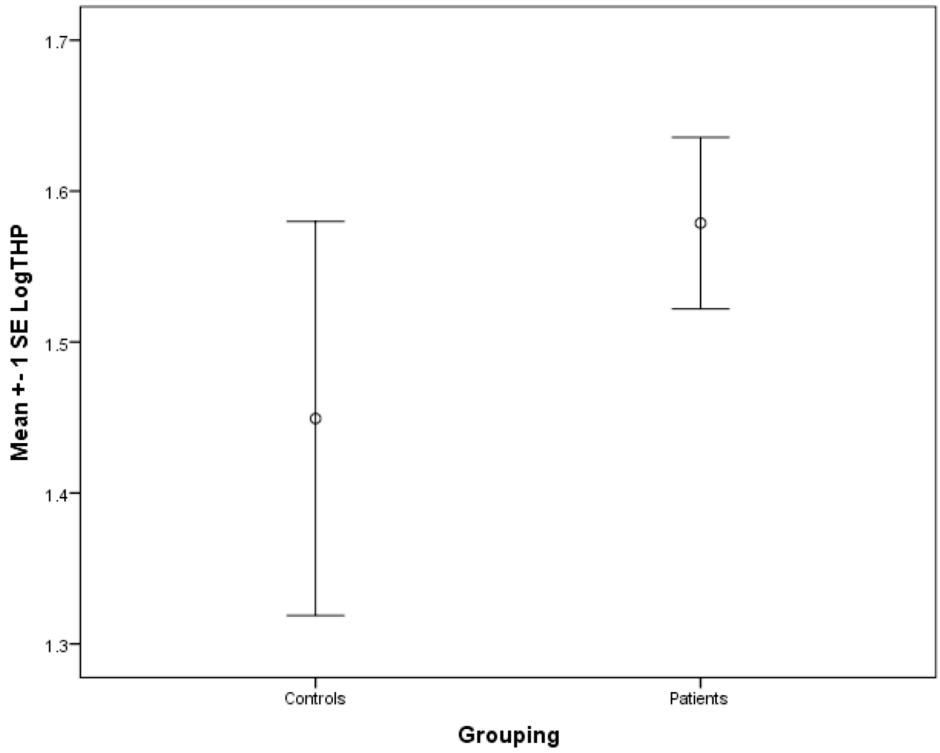
4.13.2 Statistical Analysis

The data were checked for normality using a Q-Q plot and found to be satisfactory, hence parametric methods were used for analysis. Statistical analysis of the data was conducted using ANOVA at the 95% level of confidence.

4.13.3 Results

Forty six patients (F=43; M=3; mean age (years)=40.9; sd=16.2) and 13 control subjects (F=7; M=6; mean age (years)=53.1; sd=18.0) were included in the analysis. Uromodulin was significantly different when comparing patients with OAB symptoms compared to asymptomatic controls (ANOVA F= 1.38 p=.246), as seen in figure 4.9.

Figure 4.9 - A graph showing mean Log Uromodulin and 95% confidence interval in patients with OAB symptoms and healthy controls



When comparing Uromodulin with pyuria there was no significant difference between controls or patients with varying degrees of pyuria (F=0.686, p =.418).

4.13.4 Discussion

These data do not suggest a significant difference in Uromodulin when comparing normal healthy controls with patients with OAB. However it is difficult to form strong conclusions from a small sample. In addition, there was a significant difference in mean age between controls and patients and the sample size was small. On further analysis

when comparing Uromodulin with pyuria status there was no significant difference in Uromodulin when compared to controls and patients with varying amounts of pyuria. Therefore based on this small study Uromodulin does not show potential as a marker that discriminates between controls and patients with OAB.

4.14 Monocyte Chemo-attractant Protein (MCP-1)

4.14.1 Brief description of methods

A controlled, prospective cross-sectional study of urinary monocyte chemo-attractant protein (MCP-1) in patients with OAB symptoms was undertaken. Patients with symptoms of OAB and healthy volunteers were recruited as described in chapter 2 and written consent was obtained. All participants provided clean catch MSU samples and immediate microscopy for pyuria and epithelial cells was performed. Aliquots of spun urine were frozen at -80°C, as described in chapter 2, for urinary MCP-1 quantification using a high sensitivity enzyme-linked immunosorbent assay (ELISA).

Urine samples underwent only one freeze thaw cycle to ensure stability of MCP-1. Urine samples were thawed to room temperature and mixed using a vortex mixer (Scientific Industries, New York, USA). Samples were only identifiable by study numbers to ensure blinding to sample details and group. The Quantikine Human MCP-1 Immunoassay (R and D systems) was used to measure human MCP-1 in urine. The minimum detectable dose (MDD) of MCP-1 assay was defined by the manufacture as ranging from 0.57-10.0 pg ml^{-1}. The mean MDD was 1.7 pg ml^{-1}. Seven standard solutions were prepared using the supplied MCP-1 standard (2000 pg ml^{-1}) and assay diluent to produce a 2-fold dilution series, with 2000 pg ml^{-1} standard serving as the

highest standard and Calibrator Diluent serving as the zero (0 pg ml^{-1}). Human urine samples required a 2-fold dilution using Calibrator Diluent.

A microplate containing 12 x 8 strips coated with polyclonal antibody to MCP-1 was used. Sixteen wells in the supplied microplate were filled with 200 µl of the MCP-1 standard solutions in duplicate, with the remaining wells accommodating 200 µl of each diluted urine test sample. The plate was then incubated at room temperature for 120 minutes. The well contents were then aspirated and the wells washed three times using 400µl the supplied wash solution.

After washing, 200 µL of MCP-1 Conjugate were added to each well and the plate incubated for a further 120 minutes at room temperature. The well contents were again emptied and washed three times. Two hundred µL of Substrate Solution were then added to each well and incubated for 30 minutes at room temperature protecting from light. Fifty microlitres of stop solution were then added to each of the wells with a colour change from blue to yellow and analysis undertaken within 30 minutes. MCP-1 concentration was determined using an Opsys MR fluorescence microplate reader (DYNEX Technologies, Worthing, UK), set at 450 nm. The reader produced standard curves, fit to a four-parameter logistics curve, which was used to calculate concentrates for each well. All samples were analysed in duplicate and the mean value was taken.

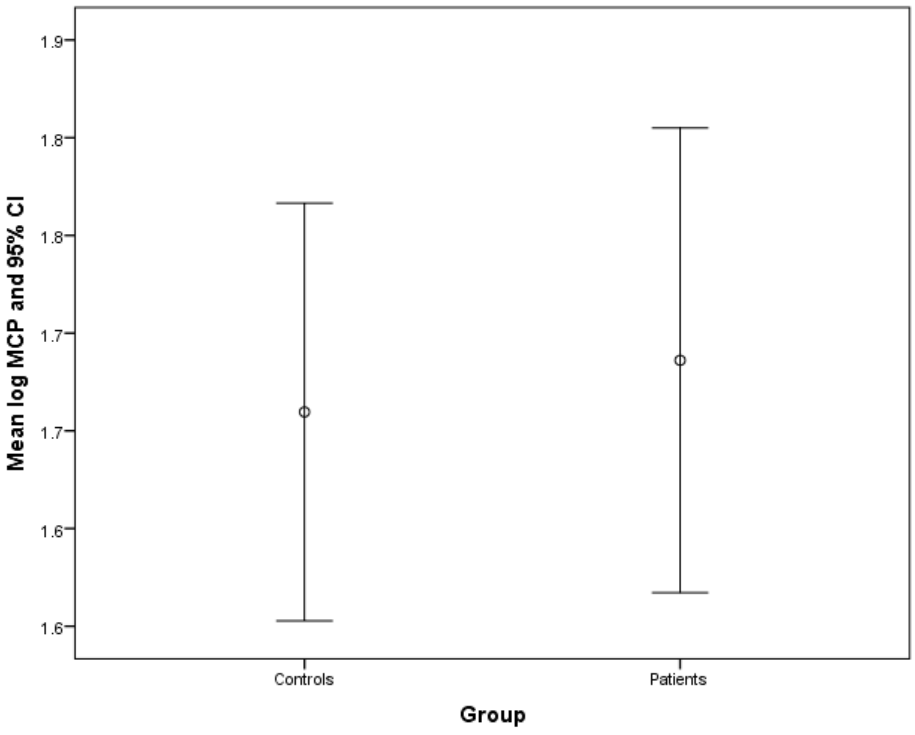
4.14.2 Statistical Analysis

The data were checked for normality using a Q-Q plot and found to be satisfactory, hence parametric methods were used for analysis. Statistical analysis of the data was conducted using ANOVA at the 95% level of confidence.

4.14.3 Results

Forty one female patients (F=41, mean age (years)=63.3; sd=11.3) and 39 female control subjects (F=39; mean age (years)=43.8; sd=16.2) were included in the analysis. When comparing OAB patients with asymptomatic controls there was no significant difference found in urinary MCP-1 (ANOVA F= 0.11 p=.740), as seen in figure 4.10.

Figure 4.10 – A Graph to show the mean Log MCP-1 and 95% confidence intervals, in patients with OAB symptoms and controls



4.14.4 Discussion

These data do not show a significant difference in urinary MCP-1 between controls and patients with OAB symptoms. Limitations of this study do include the small sample size and significant difference in age between patients and controls therefore interpretation must be taken with caution. However there does not appear to be a large difference in urinary levels of MCP-1 in patients' with OAB compared with healthy controls.

4.15 Nerve Growth Factor (NGF)

4.15.1 Brief description of methods

A controlled, prospective cross-sectional study of urinary β-Nerve Growth Factor (NGF) in patients with OAB symptoms was undertaken. Patients with symptoms of OAB and healthy volunteers were recruited as described in chapter 2 and written consent was obtained. All participants provided clean catch MSU samples and immediate microscopy for pyuria and epithelial cells was performed. Aliquots of spun urine were frozen at -80°C, as described in chapter 2, for urinary NGF quantification using a high sensitivity enzyme-linked immunosorbent assay (ELISA).

Urine samples underwent only one freeze thaw cycle to ensure stability of NGF. Urine samples were thawed to room temperature and mixed using a vortex mixer (Scientific Industries, New York, USA). Samples were only identifiable by study numbers to ensure blinding to sample details and group. Urinary NGF was measured using NGF Immunoassay (Promega) with a minimum detection of 7.8 pg ml^{-1} and less than 3% cross-reactivity with other neurotrophic factors. A standard curve was produced using 1 μg ml^{-1} NGF Standard and serial dilutions with the highest standard 250pg ml^{-1} to 3.9 pg ml^{-1}. The microplate was coated using Anti-NGF polyclonal antibody and carbonate coating buffer (pH 9.7) and incubated overnight at 4°C. The plate was then washed once and 200 μl of Block Buffer were added to each well and incubated without shaking for 1 hour at room temperature. The plate was washed once with wash buffer. Sixteen wells in the supplied microplate were filled with 100 μl of the NGF standard solutions in duplicate, with the remaining wells accommodating 100 μl of each diluted (1:2) urine sample. The plate was then sealed and incubated at room temperature for 6 hours at

37°C. The well contents were then aspirated and the wells washed five times using 200µl wash solution.

After washing, 100 µL of diluted Anti-NGF monoclonal antibody were added to each well and the plate sealed and incubated overnight at 4°C. The well contents were again emptied and washed five times. One hundred µL of Anti-Rat IgG HRP conjugate were added to each well and incubated for 2.5 hours at room temperature. The well contents were then emptied and the wells washed five times using wash solution. One hundred microlitres of TMB Solution were added to each well and incubated for 10 minutes at room temperature. The reaction was stopped by adding 100 µl of 1N hydrochloric acid to each well and a blue to yellow colour change was observed. NGF concentration was determined within 30 minutes using an Opsys MR fluorescence microplate reader (DYNEX Technologies, Worthing, UK), set at 450 nm. The reader produced standard curves, fit to a four-parameter logistics curve, which was used to calculate concentrates for each well. All samples were analysed in duplicate and the mean value was taken.

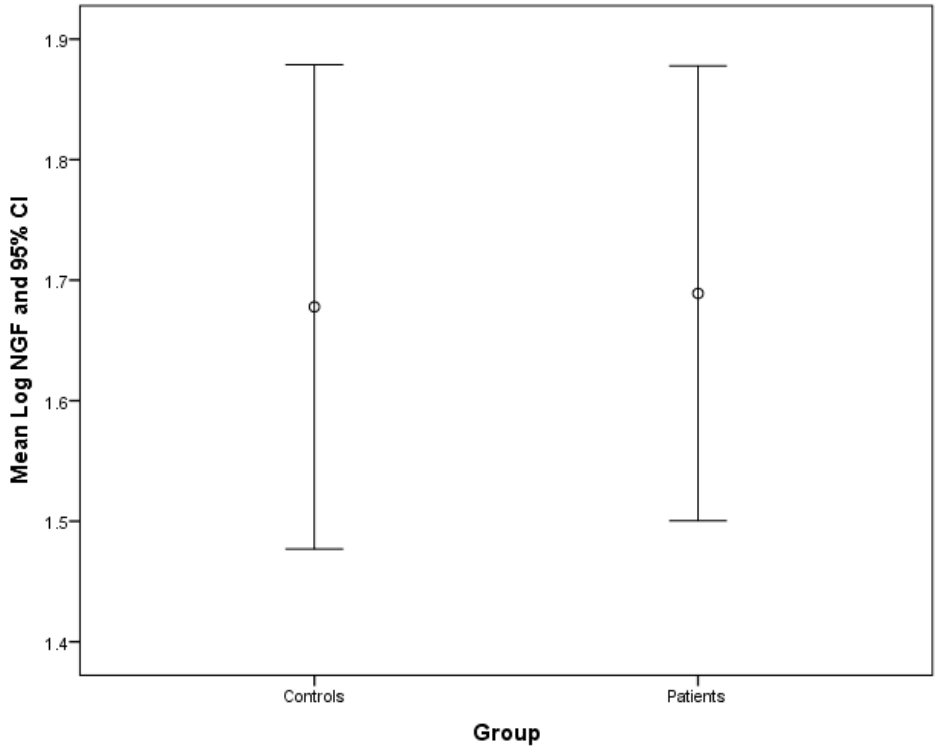
4.15.2 Statistical Analysis

The data were checked for normality using a Q-Q plot and found to be satisfactory, hence parametric methods were used for analysis. Statistical analysis of the data was conducted using ANOVA at the 95% level of confidence.

4.15.3 Results

Forty one female patients (F=41, mean age (years)=63.3; sd=11.3) and 39 female control subjects (F=39; mean age (years)=43.8; sd=16.2) were included in the analysis. When comparing OAB patients with asymptomatic controls there was no significant difference found in urinary NGF (ANOVA F= 0.007 p=.935), as seen in figure 4.11.

Figure 4.11 – A Graph to show the mean Log NGF and 95% confidence intervals, in patients with OAB symptoms and controls



4.15.4 Discussion

From this small study there does not appear to be a difference in urinary NGF when comparing patients with controls. In the literature there has been suggestion that NGF may have potential as marker of OAB hence exploration was pursued. These studies however have explored NGF as a marker of detrusor overactivity but it is well recognised that there may be other causes for symptoms of urgency. In this small study however no difference was seen between healthy controls and those with OAB symptoms. NGF also does not seem to correlate with markers of infection such as pyuria.

5 Chapter 5 – A blinded observational cohort study of the microbiological ecology associated with pyuria and overactive bladder symptoms

5.1 Hypothesis

Patients with symptoms of OAB show a discriminating urinary microbiology in urinary sediment when compared to controls.

5.2 Background

The bacterial ecology of the lower urinary tract in patients with chronic LUTS remains unexplored. Critically, the relationship between infection, inflammation, and the generation of OAB has not been scrutinised in a prospective, controlled study. This work was undertaken to characterise the ecology of the lower urinary tract using sensitive microbiological methods, and define the relationship between infection, urothelial inflammation, and symptom generation in patients OAB symptoms and healthy controls.

5.3 Study Overview

Adult female patients with OAB symptoms, and asymptomatic control subjects, were assessed at twelve study visits, scheduled every four weeks. At each visit, all study participants provided a clean catch MSU. The urine was analysed to explore the microbiology of the lower urinary tract, and the inflammatory and immune response of

the urothelium. Demographic data were stored in a secure clinical database. Symptoms were assessed using validated questionnaires.

5.4 Methods

5.4.1 Ethical review

This study was approved by the National Research Ethics Service (NRES) Committee London; East Central Research Ethical Committee 1 (Reference number 11/H0721/7).

5.4.2 Study design

This was a blinded, comparative, observational cohort study of female patients OAB symptoms, and asymptomatic control subjects using a consilience approach (figure 5.1).

5.4.3 Study Groups

Patients with OAB: Twenty female patients who describe OAB symptoms, included urinary urgency, with or without urge incontinence.

Asymptomatic controls: Twenty healthy female adults with no urinary symptoms including: urinary urgency, urinary incontinence, perception of increased urinary frequency, voiding problems, or pain attributed to the urinary tract.

All participants provided written, informed consent, and completed the ICIQ LUTS, Urgency and Pain questionnaire. Women who were pregnant or planning pregnancy were not eligible for inclusion. Any participants with an illness that in the opinion of the chief investigator may have compromised the validity of the data were excluded.

5.4.4 Recruitment of participants

Patient and control subjects were recruited as described previously in chapter 2. Additional participants were recruited to accommodate a patient dropout rate of up to 40%.

5.4.5 Data Management

Study data were stored in the Department of Medicine, UCL Archway Campus, as per Good Clinical Practice (GCP) guidance. All samples and recoded data were identifiable by study numbers and participant initials. Any patient identifiable data was stored on only a secure NHS database, which was protected by encryption and daily backup.

5.4.6 Study visits and processes

Written, informed consent was obtained at the first visit, prior to any study related procedures, and eligibility checked. Participants attended twelve study visits in total, scheduled every 4 weeks.

Patients and control subjects provided MSU samples using the clean-catch method, described in chapter 2. All samples were subject to analysis as outlined below. All participants completed symptom questionnaires.

5.4.7 Primary outcome measure

The primary outcome measure was the total log bacterial colony forming units of all isolates obtained from culture of the urinary sediment.

5.4.8 Secondary Outcome measures

The secondary outcome measures included the assessment of urothelial inflammation and immune activation, routine microbiological assessment and lower urinary tract symptoms.

Microscopic pyuria count

Urothelial cell count

Urothelial cells demonstrating associated bacteria

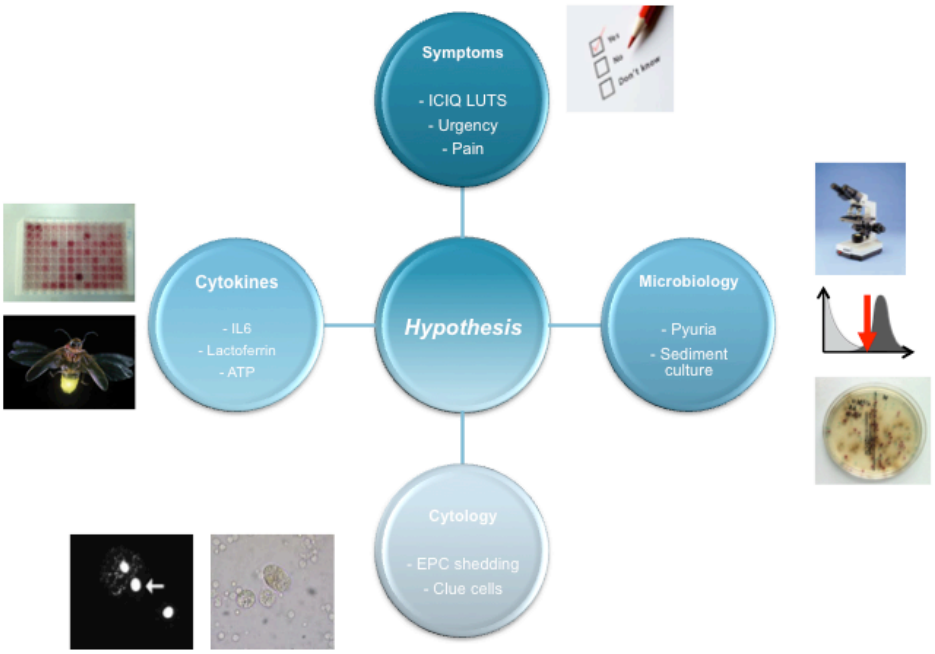
Routine urine culture in hospital laboratory

ICIQ-LUTS symptoms score

Whittington urgency score

Whittington pain score

Figure 5.1 – Study Design



5.5 Sample size calculation

A sample size of 20 participants in each group provided 83% power to detect a significant difference in log bacterial growth with alpha of 0.05. This was calculated from pilot data where the log bacterial count standard deviation was 2 and mean differcne was 0.5.

5.6 Statistical Analysis

The primary analysis was to determine the difference in total log colony forming units ml^{-1} of bacterial growth between patients and control subjects. The secondary analyses were to explore the relationship between bacterial growth, lower urinary tract symptoms and function, and markers of urothelial inflammation and immune activation. The primary outcome data were assessed for normality using Q-Q plots, and non-parametric methods of analysis employed if the data were not normally distributed. Data from patients and controls were pooled to compare the performance of routine culture methods against the results of urinary sediment culture.

The secondary analyses were undertaken using the general linear models (GLM) repeated measures procedure in SPSS. Bacterial growth was designated the dependent variable, and other measures entered as independent variables in the model. The analyses were undertaken with the supervision of a statistician familiar with the analysis of multilevel models in SPSS.

The secondary analyses were conducted to evaluate the relationship between markers of urothelial inflammation, bacterial growth, urinary symptoms, and lower urinary tract function. In addition, culture of the spun urinary sediment was used to determine the bacterial growth and routine laboratory cultures reported as positive or negative. The secondary analyses were undertaken using the linear mixed-effects models procedure in SPSS. Data were assessed for normality using Q-Q plots and log-transformed where necessary. Data that were normally distributed were analysed using parametric methods and if not normall then non-parametric tests were used.

5.7 Results

Between April 2011 and September 2013, 24 female patients with OAB (mean age=63; *sd*=11) and 22 asymptomatic control subjects (mean age 59; sd = 9) were recruited. Both groups were matched for menopausal status and BMI. There was 1 drop-out from the patient group and 1 drop-out from the control group. The results below are from the pooled data analysis of 282 patient visits and 253 control visits.

In order to examine the differences between patients and controls linear mixed-effects models procedure was used to analyse the longitudinal data with the group classification (Patient/Control) as an independent factor. Within the model the fixed effect was the group number and the dependant variables were selected in turn as LUTS score, urgency score, pain score, total bacterial growth on sediment culture, log pyuria and log epithelial cell shedding. Visit number was selected as the repeated effect. These six analyses (table 5.1) showed that group status (Patient/Control) was a significant predictor of total symptoms (Estimate of coefficient = -16.11, p<.0001); of pain (Estimate of coefficient = -1.44, p=.001); Pyuria (log pyuria) (Estimate of coefficient = -0.57, p<.0001); epithelial cell shedding (log epithelial cell count) (Estimate of coefficient = -0.30, p<.0001) and Log colony counts (Estimate of coefficient = -1.07, p<.0001).

Table 5.1 – Linear mixed models analysis with Group as the fixed effect

Dependent variable	Parameter estimate*	Significance
LUTS score	-16.11 (95% CI = -19.1 to -13.1; t= -10.8; df = 43.8)	*P<.0001*
Urgency	-6.95 (95% CI = -8.65 to -5.24; t= -8.21; df = 44.1)	*P<.0001*
Pain	-1.44 (95% CI = -2.23 to -0.64; t= -3.65; df = 44.1)	*P<.0001*
Bacterial growth§	-1.08(95% CI=-1.55 to -0.60; *t*=-4.57; *df*=41.3)	*P<.0001*
Pyuria count†	-0.57 (95% CI=-0.75 to -0.37; t=-6.37; df=41.7)	*p*<.0001
Epithelial count*	-0.30 (95% CI=-0.44 to -0.15; t=-4.21; df=43.1)	*p*<.0001

***Parameter estimate:** Increase in magnitude of dependent variable demonstrated by controls*

compared with patients subjects during the study; ***§Bacterial growth:*** *log cfu ml^{-1};* ***†Pyuria count:*** *wbc ul^{-1};* ****Epithelial count***: *epc ul^{-1};*

The linear mixed-effects models procedure was used to achieve a multiple regression, with the independent variables selected as; group number (Patient/Control), total LUTS score, urgency score, pain score, log total microbial growth and log epithelial cell shedding (table 5.2). The dependant variable was selected to be log pyuria. Visits were selected as the repeated effects. The variables that predicted log pyuria were group number, total LUTS score, log bacterial growth (Estimate of coefficient 0.14, p<.0001) and log epithelial cell shedding (Estimate of coefficient 0.25, p<.0001) and similarly the predictors of log bacterial growth were group and log pyuria (table 5.3).

Table 5.2 – Multiple mixed models analysis with log pyuria as the dependant variable

Parameter	Parameter estimate*	Significance
Group	-0.35 (95% CI = -0.09 to -0.54; t= -4.76; df = 47.5)	*P<.0001*
LUTS score	0.13 (95% CI = 0.02 to 0.01 ; t= 2.89; df = 363.6)	*P<.0001*
Bacterial growth§	0.14 (95% CI=0.17 to 0.11; *t*=8.96; *df*=483.8)	*P<.0001*
Epithelial count*	0.25 (95% CI=-0.35 to -0.15; t=5.10; df=474.9)	*p*<.0001

****Parameter estimate:*** *Increase in magnitude of dependent variable demonstrated log pyuria;;*
§Bacterial growth: *log cfu ml^{-1};* ***†Pyuria count:*** *wbc ul^{-1};* ****Epithelial count***: *epc ul^{-1};*

Table 5.3 – Multiple mixed models analysis with log bacterial growth as the dependant variable

Parameter	Parameter estimate*	Significance
Group	-0.73 (95% CI = -0.21 to -1.26; t= -2.79; df = 76.57)	*P<.007*
Pyuria count†	0.96 (95% CI = 01.15 to 0.77; t= 9.90; df = 403.43)	*P<.0001*

****Parameter estimate:*** *Increase in magnitude of dependent variable demonstrated log pyuria;*
§Bacterial growth: *log cfu ml^{-1};* ***†Pyuria count:*** *wbc ul^{-1};* ****Epithelial count***: *epc ul^{-1};*

From the pooled analysis, in contrast to controls, patients demonstrated significantly greater bacterial growth characterised by culture of the centrifuged urinary sediment ($Z=-5.981$, $p<.0001$) (figure 5.2). The median log total colony counts in the patient group were 2.30 (interquartile range 1.54) and 1.50 (interquartile range 2.01) in the control group. The patient group also showed significantly higher levels of urinary leucocyte excretion and increased clue cell shedding ($\beta=1.48$, $df=1$, $p<.0001$) (figure 5.3). In the control group the mean clue cell proportion was 0.01, median 0.00 (*sd*=0.057, interquartile range=0.00) and in the patient group mean clue cell proportion was 0.19, median 0.17 (*sd*=0.16, interquartile range=0.17).

Figure 5.2 - Total microbial growth from spun urinary sediment cultures from patients and controls

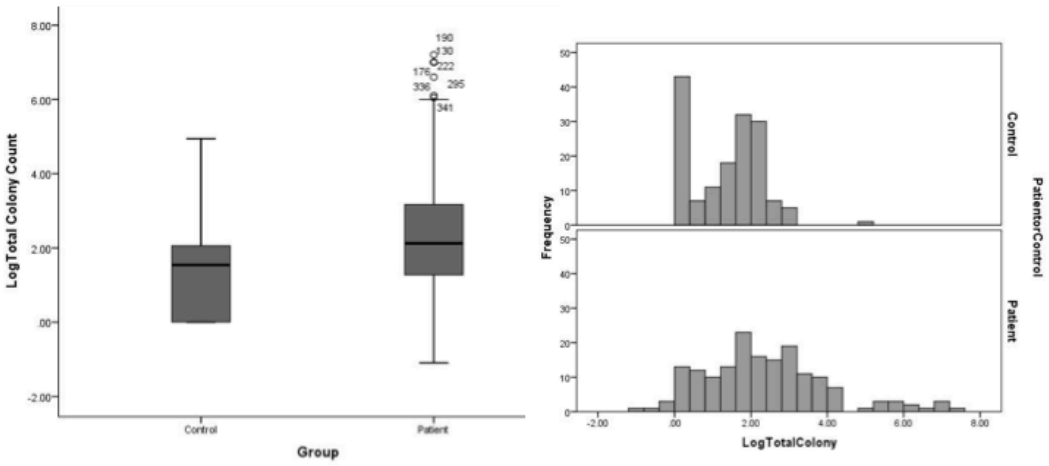
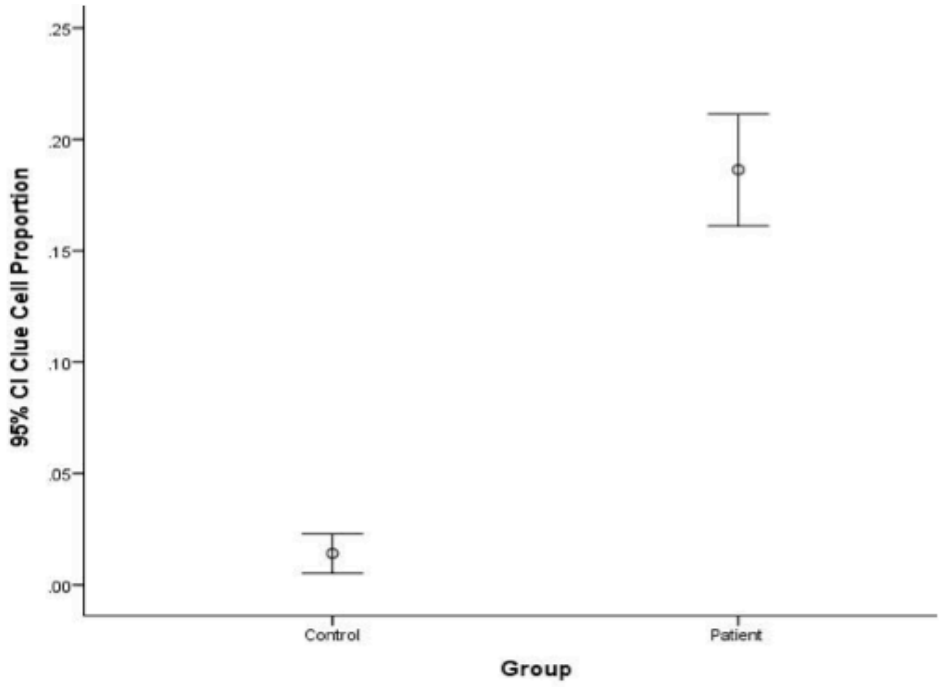


Figure 5.3 - Proportion of Clue Cells found in Patients and Controls



Within the patient group 9.72% had a positive routine culture compared with 0.40% in the control group. 93.4% of patients had a positive spun sediment culture (> 0 cfu ml^{-1}) which was significantly higher when compared to controls where 70.1% had a positive sediment culture (χ^2=51.33, p<.0001). The microbial diversity was distinctly different between patients and controls. In patient cultures, recognised uropathogens predominated (Figure 5.4).

Figure 5.4 - Microbial diversity in patients with OAB and controls

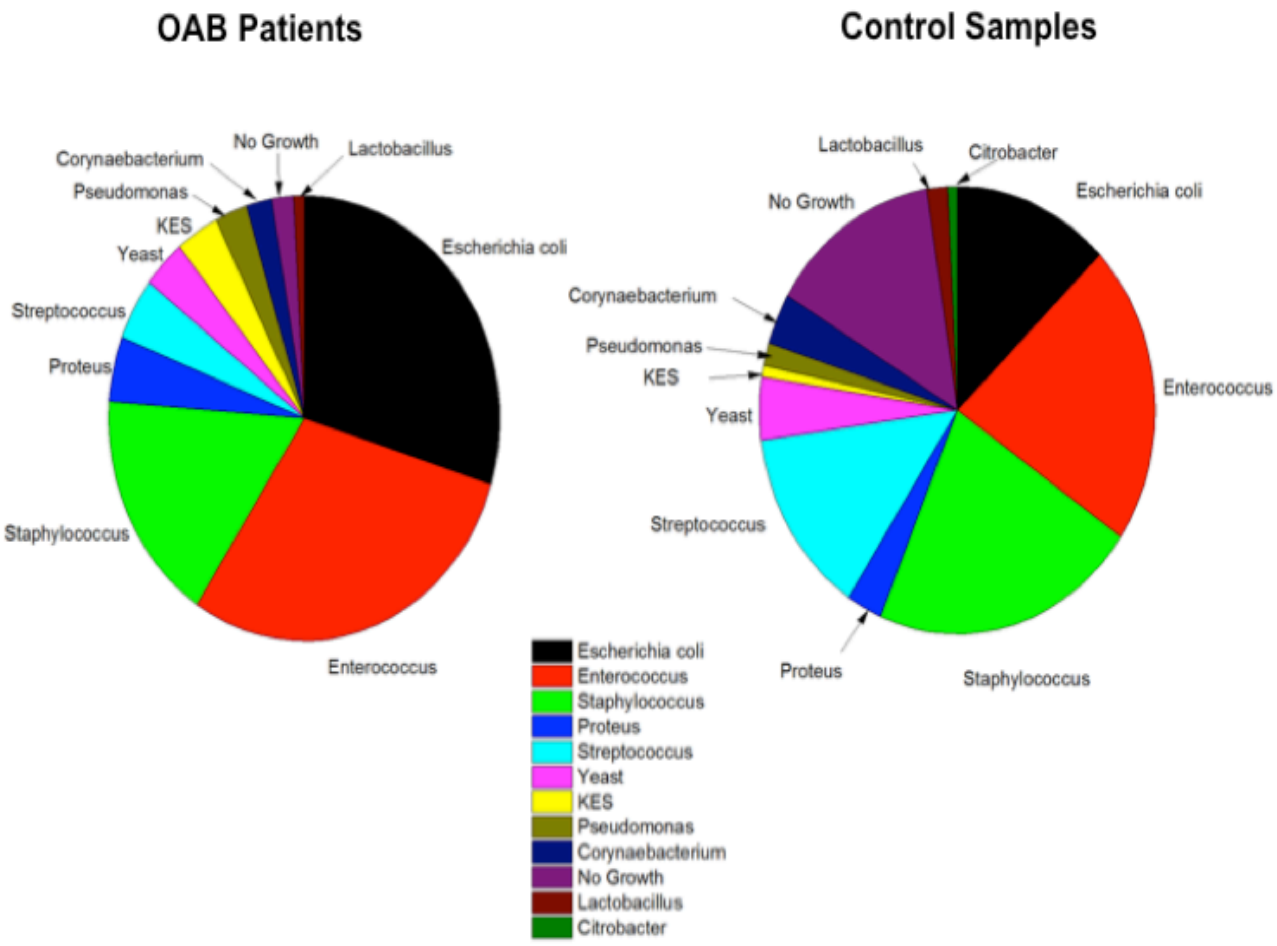


Figure 5.5 shows the distribution of microbes isolated from controls and patients. Using multinomial logistic regression, with the microbes entered into the model as factors and the dependant variable being the group (Patient/Control), *E Coli* and other coliforms and no growth proved significant predictors of the group ($\chi 2$ = 82.8, p<.0001).

Figure 5.5 – Microbial distribution between patient and controls for each organism isolated

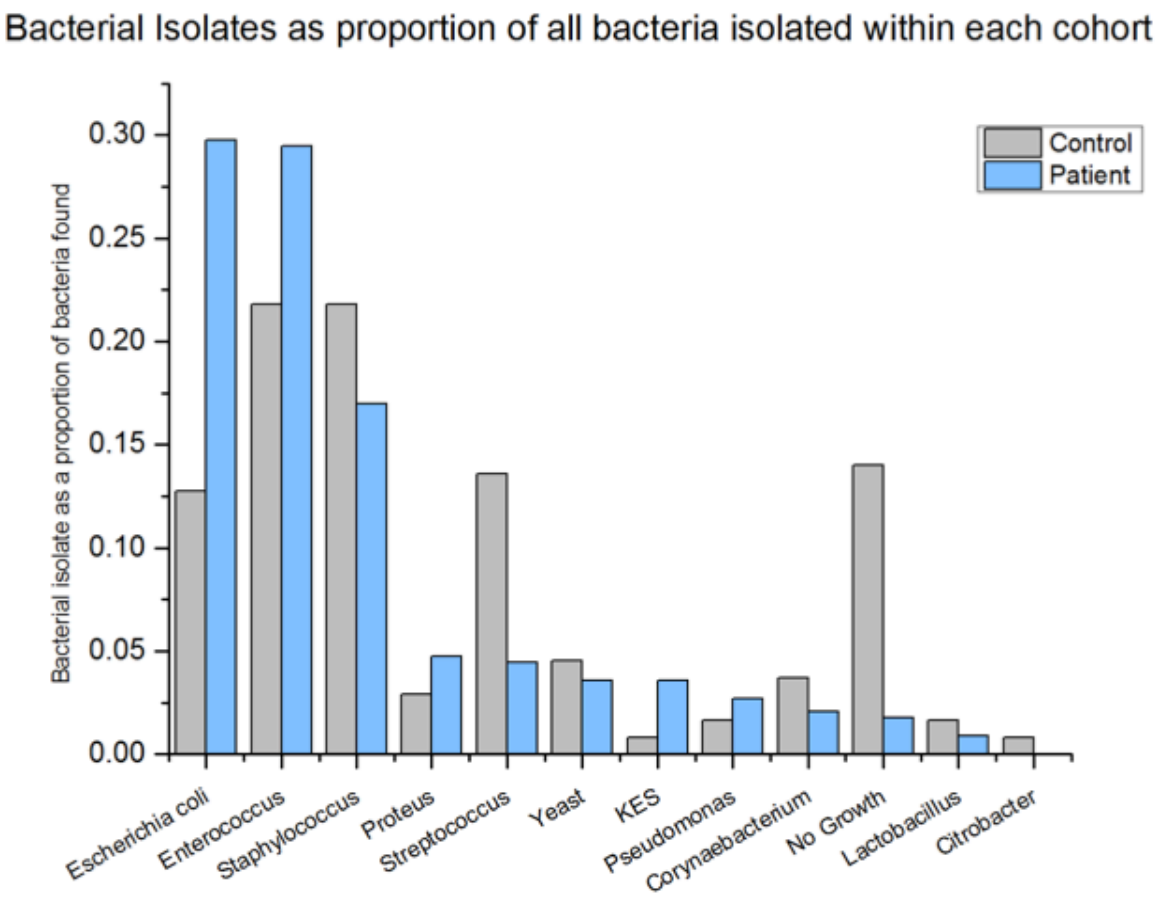


Figure 5.6 – A graph to show the proportionate distribution between patients and controls of each organism genus

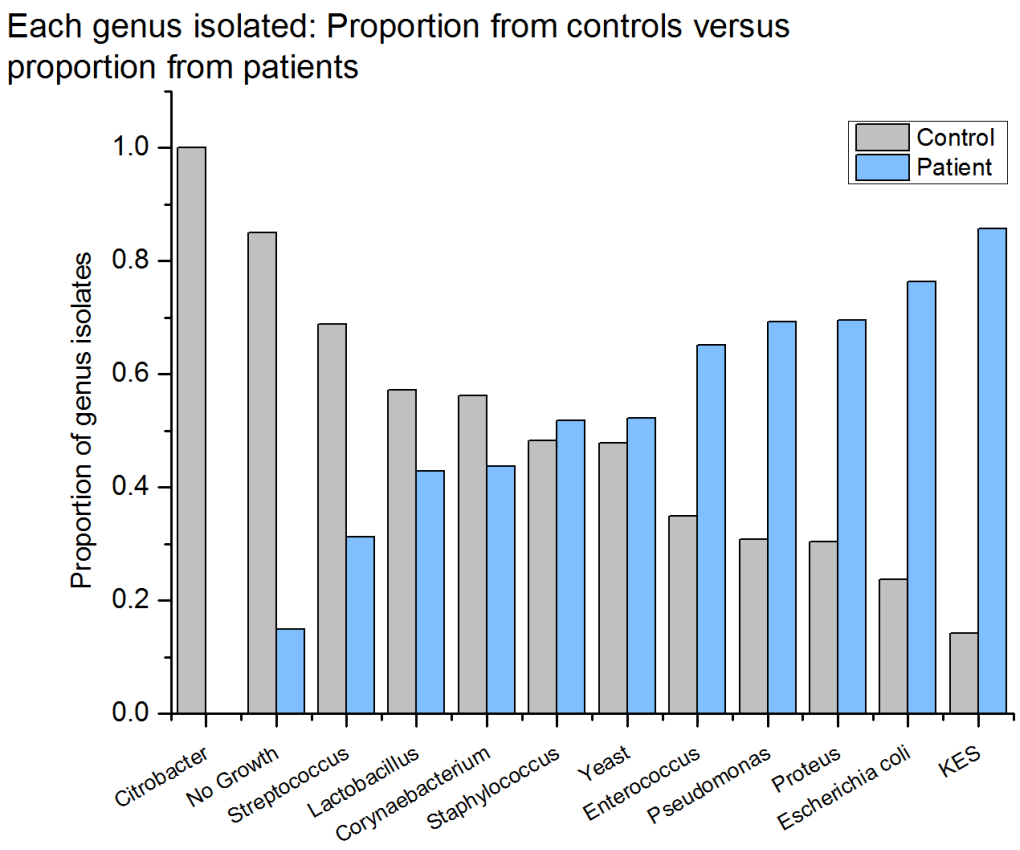
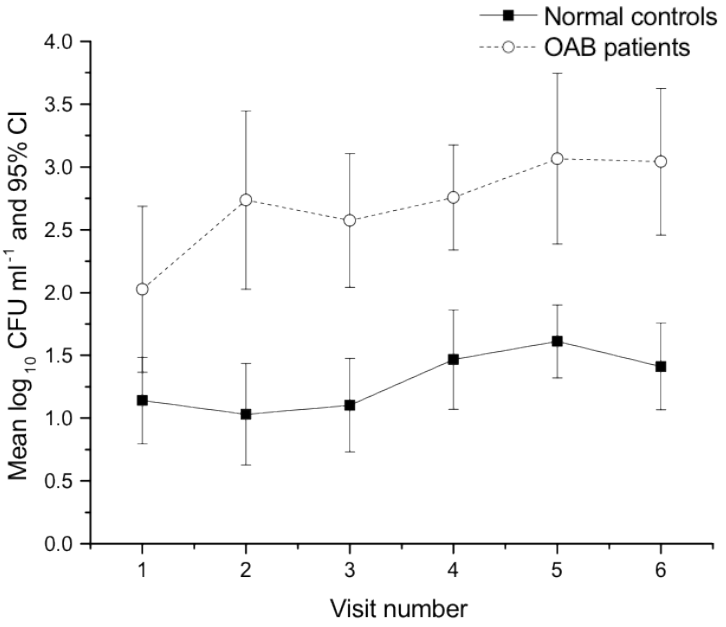


Figure 5.6 shows a proportionate occurrence, between controls and patients, of each organism genus. Thus the column pairs add to 1 in each case. There is a distinct variation in bacteria found between patients and controls. It can be suggested that some of the bacterial genus found in patients occurred less frequently in controls.

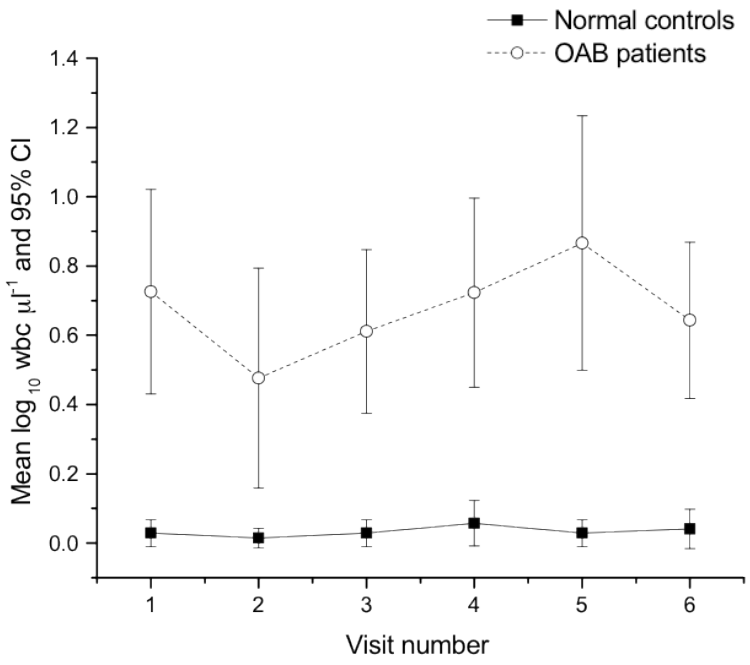
Spearman analysis demonstrated the following correlations: LUTS symptoms and pyuria (R = 0.50), urgency and pyuria (R =0.44), pyuria and epithelial cell shedding (R= 0.41). When analysing the longitudinal data, patients consistently showed a higher log bacterial growth that was significantly different to controls (figure 5.7).

Figure 5.7 – Mean Log bacterial growth in patients and controls at each visit



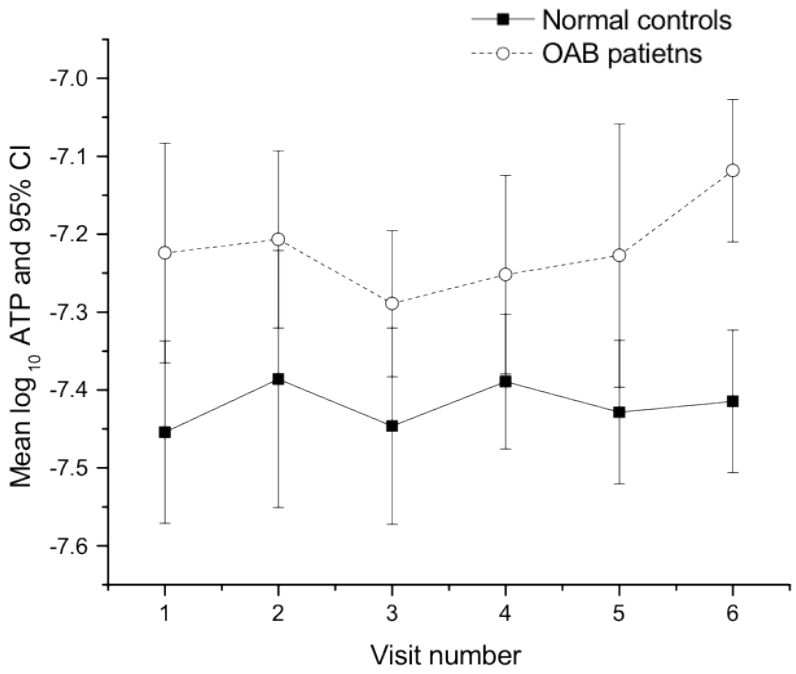
Patients also showed a significantly higher mean log pyuria when compared to controls at each visit.

Figure 5.8 – Mean Log pyuria in patients and controls at each visit



Mean urinary ATP was found to be significantly different between patients and controls across each visit (figure 5.9). The variances were considerable, but a difference was still evident though the random noise.

Figure 5.9 – Mean Log ATP in patients and controls at each visit



5.8 Discussion

This analysis of the pooled data would suggest that patients and controls appear to demonstrate differences in bacterial ecology, and differences in the expression of microscopic pyuria and clue cells. The patient group was also found to be carrying a quantitatively greater bacterial load although a diagnostic quantitative threshold is not proposed. The analysis of the longitudinal data shows a fair correlation between symptoms and pyuria, specifically urgency and pyuria and pyuria and evidence of epithelial cell shedding.

The number of participants that completed the study in the patient and control arm, taking into consideration dropouts, maintained statistical power. The study groups were matched for key demographics including age, menopausal status and BMI. Other demographics were not controlled for. The primary outcome measure of bacterial growth (cfu ml^{-1}) found through quantitative culture of the spun urinary sediment demonstrated greater bacterial growth amongst patients when compared to controls. This was in contrast to the routine laboratory culture, which did not show a significant difference in positive cultures between the two groups. These results highlight the inaccuracy of using standard culture techniques, constrained by diagnostic thresholds. These findings provide yet more evidence of the inaccuracy of fixed culture thresholds in the exploration of UTI.

The common bacterial isolates found on spun sediment in patients were different to the predominant bacterial isolates from controls. This supports work in the literature by Khasriya et al (140). *E. coli* was the most prevalent species amongst patients, followed by *Enterococcus faecalis*. *Proteus, KES and pseudomonas* were also found more commonly within the patient group as compared with the control group. Amongst control subjects, *Staphylococcus, streptococcus, Citrobacter and lactobacillus* were more commonly found.

These data suggest that *E.coli* is the most prevalent organism found on culture amongst patients, which is similar to studies that have looked at patients with acute UTI (60). *Escherichia coli* are found in the perineum and vagina and are thought to ascend and enter the urinary tract (141). Murine models have shown Uropathogenic *E. coli* to use Type 1 pilus to attach to bladder umbrella cells leading to invasion and formation of

IBCs (50, 74). The bacteria are able to release toxins that cause damage to the host and allows colonisation of the urothelium, a source of nutrients and iron for the bacteria (142) (143). The second most prevalent bacterium found in the patient cultures was *Enterococcus faecalis*. Studies have show that *Enterococcus faecalis* is able to cause damage through the release of exotoxins (144). More recently Horsley et al (101) has shown good evidence of intracellular colonisation by *Enterococcus faecalis* of urothelial cells using confocal microscopy.

Proteus spp. are thought to be opportunistic organisms and most commonly seen in urinary tract infection particularly associated with long-term catheterisation (145). Studies have also found that *Proteus mirabilis* is a key organism in biofilm formation associated with urinary catheters, causing chronic infection (146). The virulence properties of Proteus bacilli stem from flagella-mediated motility, both swimming and swarming (147) . A review by Schaffer et all described how this organism uses a diverse set of virulence factors to colonise the urinary tract, including urease and stone formation, fimbriae and adhesins, iron and zinc acquisition, proteases and toxins and biofilm formation(148).

The potential for an organism to cause disease is thought to be dependant on its virulence factors. These can be inherent to the bacteria and can also include host factors. Such virulence properties include Type-1Pilli expressed by *E.coli* or the ability to swarm by *Proteus spp*. Other factors include the ability to produce harmful toxins and the ability form intracellular bacterial communities and biofilm formation. Some uropathogens may also be capable of avoiding the immune response phagocytosis (74, 149).

The majority of patient cultures, as well as some control cultures demonstrated polymicrobial growth. Whilst mixed growth cultures have historically been associated with contamination from poor sampling, this was been strongly challenged (150, 151). Wolcott et al suggested that a polymicrobial growth and co-existent of certain species of bacteria may offer a survival advantage (152).

Urothelial clue shedding has been described as an immune response. Murine and human studies have shown increased cell shedding in response to infection (75, 76, 153). This study found that the patient group showed increased proportions of urinary clue cells, which may be a reflection of increased immune activation in response to a higher bacterial load. Conversely lower proportions of clue cells in the control group may also suggest little contamination via the clean-catch MSU sampling method. This study showed increased colonisation of shed urothelial cells in patients with LUTS compared with controls which supports work in other studies (154).

The assessment of bacterial adhesion to urothelial cells could have been affected by the sampling method. Bacterial contamination from the lower genital tract could have contributed to this. However the immediate refrigeration of urine and the processing of these samples within one hour of collection reduced this risk. Whilst one could argue that the MSU sampling method could contribute to contamination, this was appear to be nominal given the low pyuria counts and urothelial cell counts in the control population.

In the longitudinal study there was a significant difference in the microbial growth and pyuria in the patient group compared with the control group. It can be suggested that there was both an increased immune response, which may be related to bacterial growth. There is clearly a complex relationship between inflammation and bacterial proliferation that needs further exploration, the start of which has been this work. However there was also polymicrobial growth found in the control subjects, which does not trigger a host response.

A possible explanation for this may be Quorum sensing, which describes intracellular communication between bacteria. This process allows microbes to communicate and may allow modification of individual genes influencing population density and bacterial composition (155) (156). Increase in bacterial populations offers advantages over the host through virulence factors and the ability to scavenge nutrients. Bacteria use quorum sensing to coordinate behaviours including biofilm formation, virulence, and antibiotic resistance. This process is based on the local density of the bacterial population. Quorum sensing can occur within a single bacterial species as well as between diverse species. It is thought that this process is an indicator of population density or the diffusion rate of the cell's immediate environment. The mechanism of action is thought to be through secretion of secrete signalling molecules, called autoinducers by the bacteria. Common signalling molecules include oligopeptides in Gram-positive bacteria, N-acyl homoserine lactones (AHL) in Gram-negative bacteria, and a family of autoinducers known as autoinducer-2 (AI-2) in both Gram-negative and Gram-positive bacteria. These bacteria also have a receptor that can specifically detect the signalling molecule (inducer). When the inducer binds the receptor, it activates transcription of certain genes, including those for inducer synthesis. There is a low likelihood of a bacterium detecting its own secreted inducer. Thus, in order for gene

transcription to be activated, the cell must encounter signalling molecules secreted by other cells in its environment. As the population of bacterial grows, the concentration of the inducer passes a threshold, leading to the production of more inducer. This forms a positive feedback loop, and the receptor becomes fully activated (157). Activation of the receptor induces the up-regulation of other specific genes, causing all of the cells to begin transcription at approximately the same time. This coordinated behaviour of bacterial cells can be useful in a variety of situations.

These insights may start to explain the complex relationship between bacterial expansion and pyuria. Some organisms that pose potent virulence factors might attack their host at lower cell densities than microbes with lesser pathogenic potential. Quorum sensing systems are not exclusively for intra-species communication. Bacteria from different species produce universal AI molecules (158, 159).

6 Chapter 6 – The urinary cytokine and inflammatory response in relation to symptoms of OAB

6.1 Hypothesis

Patients with symptoms of overactive bladder show varying urinary inflammatory signals in relations to their symptoms.

6.1.1 Aims

To explore the variation in urinary IL6 and Lactoferrin in relation to changes in pyuria, microbial growth and symptoms in patients with OAB.

6.2 Study design and Methods

6.2.1 Ethical review

This study was approved by the National Research Ethics Service (NRES) Committee London; East Central Research Ethical Committee 1 (Reference number 11/H0721/7).

6.2.2 Study design

This was a blinded, comparative, observational cohort study of female patients with OAB symptoms, and asymptomatic control subjects.

6.2.3 Study Groups

Female patients who described OAB symptoms, including urinary urgency, with or without urge incontinence. Asymptomatic controls: healthy female adults with no urinary symptoms including: urinary urgency, urinary incontinence, perception of

increased urinary frequency, voiding problems, or pain attributed to the urinary tract. Recruitment was as described in chapter 2.

Subject Inclusion Criteria

9. Adults Women aged >=18 years
10. Able to complete a symptom questionnaire
11. Diagnosed with OAB with or without pyuria (≥ 1 wbc μl^{-1})
12. Post-menopausal women or women using adequate contraception

Subject Exclusion Criteria

9. Age less than 18 years
10. Inability to consent
11. Pregnant women or women planning to conceive
12. Patients with concurrent illnesses that in the opinion of the investigator are likely to compromise the validity of the data

The study groups were:

1) Female adult patients with OAB, describing:

f. Urgency, the sudden compelling desire to urinate, a sensation that is difficult to defer.
g. Urinary frequency, voiding eight or more times in a 24-hour period.
h. Nocturia, the need to wake one or more times per night to void.
i. They may or may not experience urinary incontinence.
j. With or without pyuria (≥1 wbc μL^{-1}) and/or a history of acute symptom exacerbations.

2) Female adults without OAB and without other lower urinary tract symptoms.

6.2.4 Recruitment of participants and consent

Patients were recruited from urological clinics at the Department of Medicine, University College London Archway Campus. The patients who expressed interest were given written information regarding the study and then contacted two weeks later and if agreeable, invited for an initial visit where written informed consent was obtained. Control subjects were recruited from the staff at the Whittington Hospital and University College London.

6.2.5 Data Management

Study data were stored in the Department of Medicine, UCL Archway Campus, as per Good Clinical Practice (GCP) guidance. All samples and recoded data were identifiable by study numbers and participant initials. Any patient identifiable data was stored on only a secure NHS database, which was protected by encryption and daily backup.

6.2.6 Study visits and processes

Written, informed consent was taken at the first visit, prior to any study related procedures, and eligibility checked. Participants attended twelve study visits in total, scheduled every 4 weeks. The study design can be seen in figure 6.1. All participants completed symptom questionnaires including ICIQ-FLUTS, Whittington urgency score and Whittington pain score. Patients and control subjects provided MSU samples using the clean-catch method. All samples were subject to analysis by blinded researchers. Immediate microscopy for pyuria and epithelial cells was performed. Aliquots of spun

urine were frozen at -80°C. These process and methods are all described in detail in chapter 2.

6.2.7 Primary outcome measure

The primary outcome measure was urinary IL6 and urinary Lactoferrin in patients with OAB symptoms and asymptomatic controls.

6.2.8 Secondary Outcome measures

The secondary outcome measures included the assessment of urothelial inflammation and immune activation, routine microbiological assessment and lower urinary tract symptoms.

Microscopic pyuria count

Urothelial cell count

Urothelial cells demonstrating associated bacteria

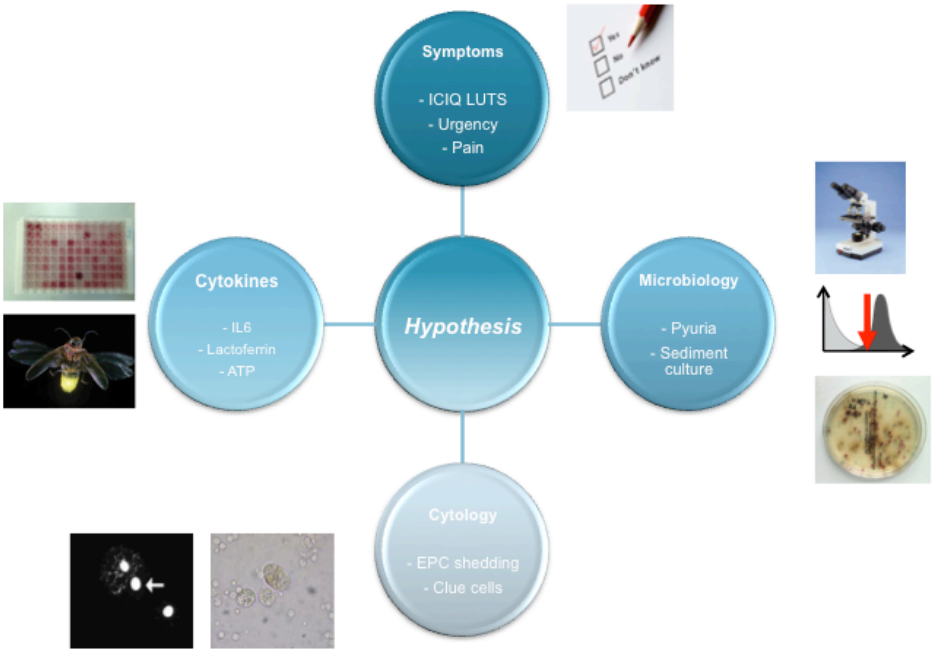
Microbial growth using enhanced sediment culture

ICIQ-LUTS symptoms score

Whittington urgency score

Whittington pain score

Figure 6.1 – Study Design



6.3 Additional Methods

6.3.1 Quantification of Urinary IL6

The samples were thawed to room temperature and analysed in batches, and blinding to sample details and symptoms ensured. The Quantikine® High Sensitivity ELISA Human IL6 Immunoassay was used to quantify urinary IL6 (R&D Systems, Abingdon, UK), with a limit of detection of 0.09pg ml^{-1} with an inter- and intra-assay coefficient of variation of less than 10%. Frozen urine samples under went only one freeze thaw cycle ensuring stability of IL6. The urine samples were thawed to room temperature and mixed thoroughly using a vortex mixer (Scientific Industries, New York, USA). The assay included IL6 stock standard at a concentration of 10 pg ml^{-1}. Calibrator diluent was used to produce serial dilutions ranging from 10 pg ml^{-1} to 0.156 pg ml^{-1}. Calibrator diluent was used as 0 pg ml^{-1} standard.

The supplied pre coated 96 well microplate was used to plate the IL6 standards and urine samples. 100 µl of the eight IL6 standards were plated in duplicate. 40 urine samples were plated in duplicate in the remaining 80 wells by adding 100 µl of each urine sample. The plate was then covered and incubated at room temperature. Binding was encouraged using an orbital microplate shaker (0.12" orbit) set at 500 ± 50 rpm for two hours.

After the 2 hour incubation the well contents were remove and the wells washed six times with wash solution. Each wash was carefully pipetted without disruption to the base of the well. In a second incubation, 200µl of IL6 conjugate was then added to each well, and the plate incubated for a further two hours. The well contents were washed as described earlier. The plate was then incubated on the bench top, after addition of 50µl of substrate solution, at room temperature for one hour. Following this was the addition of 50 µl of amplifier solution without any washing and a colour change noted. The plate was incubated on the bench top at room temperature for 30 minutes. In the final step 50µl of stop solution was added followed by analysis within 30 minutes. Interleukin-6 concentration was determined using an Opsys MR fluorescence microplate reader (DYNEX Technologies, Worthing, UK), set at 490 nm. The reader produced standard curves, which were used to calculate concentrates for each well. The minimum detectable dose (MDD) of the ELISA kit according to the manufacturers ranges from 0.016-0.110 pg ml^{-1}, with an average MDD of 0.039 pg ml^{-1}. All samples were analysed in duplicate to test the inter-assay precision and the mean value was taken. The intra-assay precision for urine assays suggested by the company was 5.5-9.8% and the inter-assay precision for this experiment was 5.5-11.2%.

6.3.2 Quantification of Urinary Lactoferrin

Urine samples underwent one freeze thaw cycle to ensure stability of Lactoferrin. Urine samples were thawed to room temperature and mixed using a vortex mixer (Scientific Industries, New York, USA). Samples were only identifiable by study numbers to ensure blinding to sample details and group. The Human Lactoferrin ELISA immunoassay was used to quantify urinary Lactoferrin (ICL, Portland, USA), with a range of detection of 3.125ng ml^{-1} -100ng ml^{-1}, with a sensitivity average of 0.725ng ml^{-1}. Lactoferrin standard solutions were prepared using 100 ng ml^{-1} stock lactoferrin standard and six serial dilutions using calibrator diluent. This produced standard solutions between 100ng ml^{-1} and 3.125ng ml^{-1}, and calibrator diluent was used as zero standard.

Fourteen wells in the supplied microplate were filled 100 µl of the seven Lactoferrin standard solutions in duplicate, with the remaining wells accommodating 100 µl of each urine test sample. The plate was incubated on the bench top at room temperature for 30 minutes to allow binding. Following incubation well contents were aspirated and the wells carefully filled with wash solution. This wsa then carefully aspirated and the process repeated 3 times.

After washing, 100µl of Enzyme Antibody Conjugate were added to each well, and the plate incubated for a further 30 minutes. The well contents were again emptied and washed four times. One hundred microlitres of the supplied substrate solution was then added to each well, and the plate incubated in a dark room at room temperature for 10 minutes. One hundred microlitres of the supplied stop solution were then added to each of the wells and analysis undertaken within 30 minutes. Lactoferrin concentration

was determined using an Opsys MR fluorescence microplate reader (DYNEX Technologies, Worthing, UK), set at 450 nm. The reader produced standard curves, fit to a four-parameter logistics curve, which was used to calculate concentrates for each well. All samples were analysed in duplicate to test the inter-assay precision and the mean value was taken.

6.4 Sample size calculation

The study was powered to detect a clinically significant difference between groups in terms of microbial culture colony count. For any surrogate marker of inflammation or infection we should expect discriminatory power at least as effective as colony counts. This study was therefore powered to function within those strictures. Pilot data demonstrated that the standard deviation of the log bacterial count was 2 (sd=2.0), and the standardised mean difference in log bacterial counts was 0.5 (f=0.5). A sample size of 20 subjects in each group would provide 83% power to detect a significant difference in log bacterial growth at the 5% level (α=0.05).

6.5 Statistical Analysis

The primary statistical analysis was to determine the difference in urinary IL6 and Lactoferrin in patients compared to controls. The secondary analyses were to explore the relationship between these urinary markers of inflammation and bacterial growth, lower urinary tract symptoms and function. The primary outcome data were assessed for normality using Q-Q plots, and parametric methods of analysis used as the data were normally distributed. Data from patients and controls were pooled to compare the performance of urine cytokines in relation to pyuria, bacterial growth by enhanced sediment culture and symptoms, in patients and controls. A linear mixed-effects models

analysis was used to analyse the data to take into account the repeated measures. This was undertaken using the linear mixed-effects models procedure in SPSS. Urinary IL6 and Lactoferrin were designated as the dependent variables, and other measures entered as independent covariates in the model. The analysis was undertaken with the supervision of a statistician familiar with the analysis of multilevel models in SPSS.

6.6 Results

Between April 2011 and September 2013, 24 female patients with OAB (mean age=63; *sd*=11) and 22 asymptomatic control subjects (mean age 59; sd = 9) were recruited. Both groups were matched for menopausal status and BMI. There was 1 drop-out from the patient group and 1 drop-out from the control group. These results are from the pooled data from 144 OAB patient visits and 136 control visits.

The linear mixed-effects models procedure was used to analysis the longitudinal data and explores the relationship between urinary IL6 and urgency score, pain score, LUTS score, pyuria and microbial growth on enhanced sediment culture. In the first model the independent covariate was the group (0 or 1 / control or patient), the dependant variable IL6 and the repeated variables were indexed on visit number. There was a significant difference in urinary IL6 between patients and controls (Table 6.1).

Table 6.1 – Linear mixed models analysis with IL6 as the dependant variable

Parameter	Parameter estimate*	Significance
Group	16.1 (Mean = -0.24 95% CI = -0.31 to -0.57; t= -6.8; df = 42.2)	*P<.0001*

***Parameter estimate:** Increase in magnitude of dependent variable demonstrated group (control = 0 and patient = 1)*

In the second model the dependant variable was log IL6 and the independent covariates were the group number (0/1), urgency score, pain score, LUTS score, log pyuria and log total microbial growth. The repeated variables were indexed on visit number. Table 6.2 shows the results; it can be seen that pyuria count (log_{10} wbc μl^{-1}) proved a significant predictor of IL6 however total bacterial growth (log_{10} CFU ml^{-1}), epithelial cell count (log_{10} epc μl^{-1}) and the symptoms scores did not.

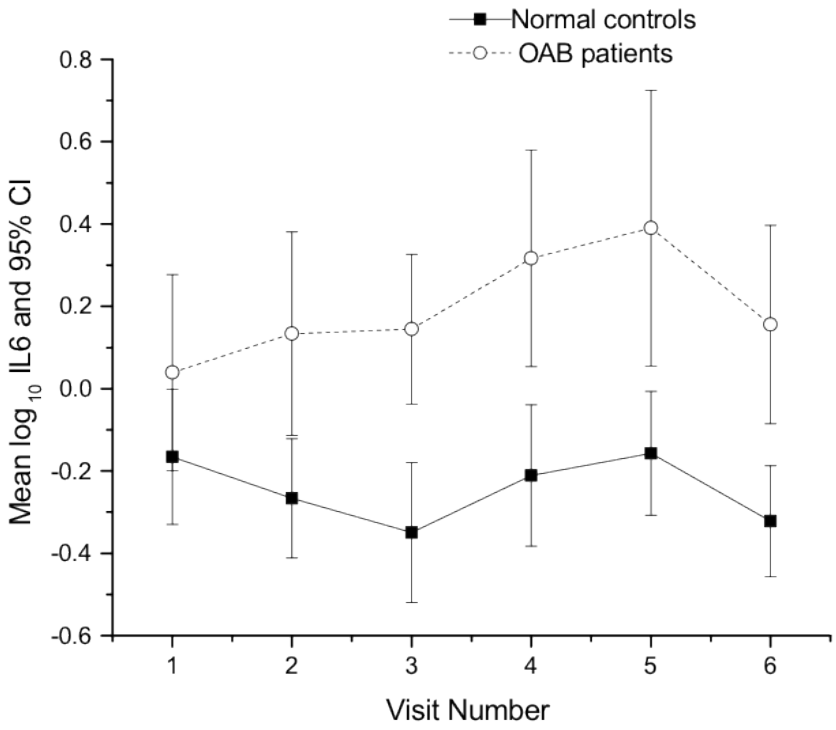
Table 6.2 - Mixed models analysis with IL6 as the dependant variable

Dependent variable	Parameter estimate*	Significance
Group	0.12 (F = 2.34; sd = 0.03; df = 57.4)	*P=.13*
LUTS score	0.09 (F = 0.24; sd = 0.02; df = 79.0)	*P=.63*
Urgency	0.08 (F = 0.16; sd = 0.02; df = 213.2)	*P=.69*
Pain	0.16 (F = 0.76; sd = 0.04; df = 182.4)	*P=0.39*
Bacterial growth§	0.08 (F = 2.64; sd = 0.02; df = 227.9)	*P=.11*
Pyuria count†	**0.18 (F = 64.4; sd = 0.04; df = 237.0)**	***P*=<.0001**
Epithelial count*	0.13 (F = 0.05; sd = 0.03; df = 227.9)	*P*=.106

***Parameter estimate:** Increase in magnitude of dependent variable demonstrated by patients compared with control subjects during the study; **§Bacterial growth:** log cfu ml^{-1}; **†Pyuria count:** wbc ul^{-1}; ***Epithelial count**: epc ul^{-1};*

Mean urinary IL6 was found to be significantly higher in patients when compared with controls across each visit (figure 6.2).

Figure 6.2 – Mean Log IL6 in patients and controls at each visit.



The linear mixed-effects models procedure was used to analyse the longitudinal data and explore the relationship between urinary Lactoferrin and urgency score, pain score, LUTS score, pyuria and microbial growth on enhanced sediment culture. In the first model the independent covariate was the group (0 or 1 / control or patient), the dependant variable Lactoferrin, and the repeated variables were indexed on visit number. There was a significant difference in urinary Lactoferrin between patients and controls (table 6.3).

Table 6.3 – Linear mixed models analysis with Lactoferrin as the dependant variable

Parameter	Parameter estimate*	Significance
Group	51.8 (Mean = -1.27 95% CI = -1.10 to -1.44; t= -6.8; df = 42.7)	*P<.0001*

***Parameter estimate:** Increase in magnitude of dependent variable demonstrated group (control = 0 and patient = 1)*

A multiple mixed-effects models analysis was performed where the dependant variable was log Lactoferrin and the independent covariates were the group number (0/1), LUTS score, urgency score, pain score, log total microbial growth and log pyuria. Table 6.4 shows the results for each of these parameters. The predictors of Lactoferrin proved to be the total bacterial growth (log_{10} CFU ml^{-1}), pyuria count (log_{10} wbc μl^{-1}) however epithelial cell (log_{10} wbc μl^{-1}) count and symptoms did not.

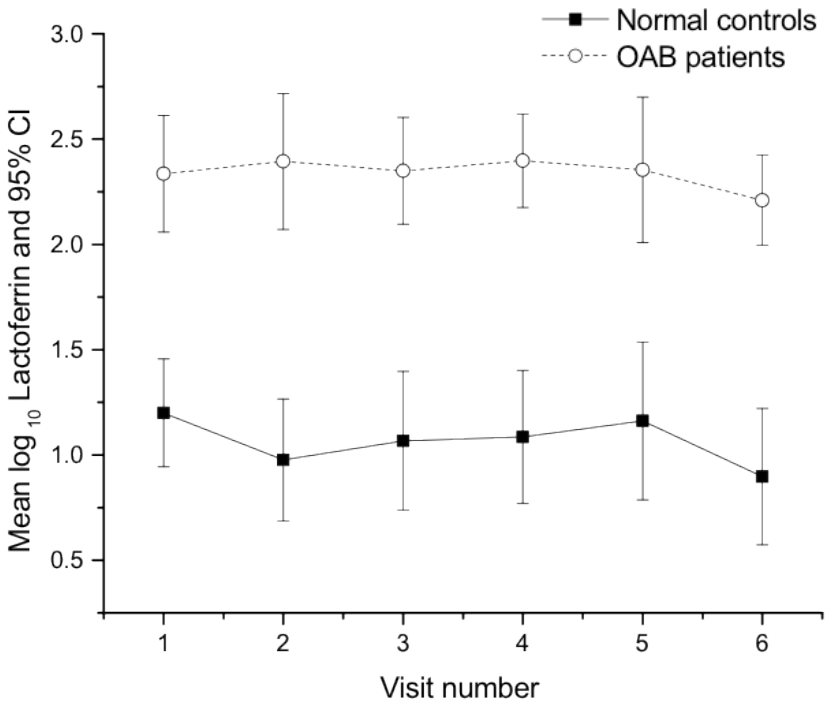
Table 6.4 - Mixed models analysis with Lactoferrin as the dependant variable

Dependent variable	Parameter estimate*	Significance
Group	**0.10 (F = 25.19; sd = 0.03; df = 70.6)**	***P=<.0001***
LUTS score	0.14 (F = 1.67; sd = 0.02; df = 180.0)	*P=.198*
Urgency	0.11 (F = 0.08; sd = 0.02; df = 228.1)	*P=.782*
Pain	0.07 (F = 3.89; sd = 0.04; df = 178.3)	*P=.050*
Bacterial growth§	**0.27 (F = 4.54; sd = 0.02; df = 201.5)**	***P=.034***
Pyuria count†	**0.25 (F = 56.65; sd = 0.04; df = 179.4)**	***P=<.0001***
Epithelial count*	0.11 (F = 0.001; sd = 0.03; df = 187.7)	*P=.970*

***Parameter estimate:** Increase in magnitude of dependent variable demonstrated by patients compared with control subjects during the study; **§Bacterial growth:** log cfu ml^{-1}; **†Pyuria count:** wbc ul^{-1}; ***Epithelial count**: epc ul^{-1};*

Mean urinary Lactoferrin was found to be significantly higher in the patient group when compared with controls across each visit (figure 6.3).

Figure 6.3– Mean Log Lactoferrin in patients and controls at each visit.



Between log pyuria and log bacterial growth there was a correlation of 0.61. There was a correlation of 0.63 between log IL6 and log pyuria. A similar correlation of 0.67 was found between log pyuria and log Lactoferrin. Between log IL6 and log Lactoferrin there was a correlation of 0.58.

6.7 Discussion

This is first prospective study that has addressed the role of infection and inflammation in the generation of OAB symptoms. From cross-sectional data there is a growing body of evidence that bacterial infection may play a role in the LUTS and OAB. In this study the patient group and control were matched for age, BMI and menopause status and statistical power was maintained given the sample size. There was a significant difference in the microbial growth, pyuria, urinary IL6 and Lactoferrin in the patient

group compared with the control group. In the longitudinal study there was a significant difference in the microbial growth and pyuria in the patient group compared with the control group.

Urinary IL6 had an association with pyuria. Increased urinary IL6 secretion has been demonstrated in prospective studies of acute UTI (93). Bacteria are able to trigger an innate immune response by activating epithelial cells. Like other mucosal surfaces, the lining of the urinary tract contains receptors capable of recognizing intruding pathogens by pathogen associated molecular patterns (PAMPS). Of the various immune surveillance molecules the Toll-like receptor (TLR) family is the best characterized. Mechanisms proposed involve bacterial adherence and TLR4 signalling. In vivo studies where there has been a bacterial challenge to urothelial cells, a rapid cytokine response was observed, with production of interleukin-1beta (IL-1beta), IL-6, and IL-8. In this study type 1-fimbriated E. coli activated IL-6 and IL-8 production more efficiently than the non-fimbriated (Samuelsson 2004). IL-6 is detected in the urine at the time of diagnosis in most patients with bacteria in the urinary tract, however, IL-6 was only detected in the serum of symptomatic patients (91) . These observations suggested that the cytokine response during UTI could have local and systemic components. Epithelial cells are thought to be responsible for the local cytokine response (88, 100, 160). Studies have shown that IL6 production increases neutrophil migration and activation of IL8 (161, 162). Adhering bacteria and isolated P fimbriae stimulate higher levels of IL-6 production in cells that express the globoseries of glycolipids such as kidney epithelial cells. Uroepithelial cells also respond to stimulation by cytokines; IL-1alpha and TNFalpha induce the secretion of IL-6 and IL-8 and the upregulation of mRNAs for IL-1alpha, IL-1beta, IL-6 and IL-8 (91, 163).

Further data from prospective studies of UTI are needed to improve our understanding of its role in infection. There is a complex regulation of the production of Interleukin-6 by membrane-bound and soluble receptors, which are modulated by a host of other influences (164). Strains of UPEC have developed strategies to evade immune response by suppressing cytokine reactions to infection (149, 165, 166). Whilst the onset of acute bacterial cystitis appears to be associated with an increase in urinary IL6 secretion (93), this study would suggest it has a role in chronic infection.

Urinary Lactoferrin was associated with pyuria and microbial growth. Lactoferrin is a functional protein of the transferring family. It is part of our innate immune defence at mucosal surfaces. Lactoferrin's primary role is to sequester free iron. Iron is a nutrient essential for bacterial growth and survival. Hence in doing so, Lactoferrin competes directly with bacteria and removes an essential substrate needed for bacterial growth (167). It can therefore be proposed that when there is an increase in bacterial load, urinary Lactoferrin would be elevated. It has also been proposed that the antibacterial action of Lactoferrin is explained by the presence of specific receptors on the cell surface of microorganisms. The mechanism for this is via binding to the lipopolysaccharide (LPS) of the bacterial wall and the oxidation of the bacteria via formation of peroxides. This affects the membrane permeability and results in the cellular lysis (167).

Lactoferrin has other antibacterial mechanisms not related to iron, such as stimulation of phagocytosis. However it is thought that the dominant mechanism of action is via its iron binding properties and interaction with LPS of the bacterial cell wall (168, 169).

Lactoferrin's binding to the bacteria wall is associated with the specific peptide lactoferricin, which is located at the N-lobe of Lactoferrin and is produced by in vitro cleavage of Lactoferrin with trypsin (170, 171). The antimicrobial action of Lactoferrin has also been reported via targeting H+-ATPase and interference with proton translocation in the cell membrane, resulting in a lethal effect in vitro, as well as preventing attachment (172).

Bortner et al. (1989) proposed that this iron-independent Lactoferrin killing was a result of a direct interaction of Lactoferrin with the bacterial surface (173). Lactoferrin has been shown to interact with LPS of the Gram-negative bacterial membrane of *E. coli*, with the release of the LPS from the membrane (174). However, this release was blocked by addition of Ca2+ and Mg2+ ions, suggesting the importance of these divalent cations as modulators for the antimicrobial activity of Lactoferrin (173). Lactoferrin sensitive strains of Legionella pneumophila were protected from killing by addition of calcium chloride, magnesium nitrate and magnesium chloride, however addition of sodium chloride had no effect (173). Kalmar et al showed that Mg2+ ions decreased Lactoferrin killing, whereas the addition of Ca2+ or K+ ions had no affect on the antimicrobial activity of Lactoferrin (175).

Studies have suggested that Lactoferrin binds to the isolated lipid A portion of LPS (174). The N-terminal region of Lactoferrin, which includes the lactoferricin peptide sequence, was shown to be involved in the binding of intact Lactoferrin to the LPS of *E. coli* 055B5 (176). Lactoferrin has also been shown to interact reversibly with porins of the *E. coli* membrane. However these porins may be shielded from attack by the polysaccharide portion of the LPS hence decreasing antibacterial activity (177). The susceptibility of the bacteria to Lactoferrin is thought to be dependent on their growth

phase since bacteria are more susceptible to killing by Lactoferrin when in early phase (173, 175). The binding of Lactoferrin to these bacteria was temperature sensitive with no binding at 4°C. Although binding was shown to be independent of killing, a decrease in the pH of the media increased the sensitivity of the bacteria (173). Lactoferrin has also been shown to modulate the activity of known antibacterial agents such as lysozyme and antibiotics, with the potential of working synergistically (178, 179).

These data are the first prospective study using a consilience approach, to demonstrate an association between bacterial infection, markers of urothelial inflammation and the overactive bladder symptoms. This work has helped to explore the role of infection and inflammation in patients with symptoms of overactive bladder, however more work is needed to improve our understanding of the precise mechanisms involved in the generation of such symptoms.

7 Chapter 7 – General Discussion and Conclusions

Exclusion of a urinary tract infection is a universal mandatory first step in the management of patients with lower urinary tract symptoms. Serious concerns have been raised about the performance of current diagnostic tests commonly used to exclude UTI (40, 42, 43, 48, 180, 181). These tests have been found to perform so poorly that we should doubt whether urinary infection was ever reliably excluded in the studies that characterised OAB. Thus it is appropriate to re-visit this common condition and scrutinise it carefully for evidence of infection or inflammation that may have been missed in the past. A role for infection in the generation of LUTS and OAB symptoms has been postulated in recent times (101, 102, 182, 183). This study explored the presence of infection and inflammation in patients with LUTS and OAB symptoms. Data were collected using a comparative method that included asymptomatic normal controls. These were used to determine whether patients with OAB symptoms demonstrated evidence of urinary infection or inflammation that would be missed by usual routine testing. The relationship between bacterial infection, urinary inflammation and OAB symptoms was also explored as part of a consilience approach. Since urinary ATP has been promoted as a potential test for OAB and for UTI, this proposition was investigated. However, there was no intention of exploring any of the other markers as potential clinical tests. The history of this subject contains numerous examples of the problems of co-opting a novel pathological observation in order to design a clinical test. The pathological manifestations of disease are more valuable for informing our understanding.

7.1 Limitations and weaknesses

Patients with overactive bladder symptoms were recruited along with controls to explore the presence of infection and inflammation using a number of independent markers of infection. Symptoms were measured using three different validated tools but quality of life (QOL) was not included. Lower urinary tract symptoms and OAB symptoms are more common in females although significant numbers of men also suffer. In the longitudinal study only female patients were included. Although the total number of patient samples was large, the numbers were insufficient to permit any sub-group analyses.

All patients and controls provided clean catch midstream urine samples. The use of catheter sampling in subjects would have been unacceptable, particularly as the data were collected prospectively over multiple visits. Clear direction and a specific protocol were used to minimise contamination however some contamination may have occurred.

The culture methods used in this work were favourable for growth of aerobic uropathogens. Culture conditions and techniques for detection of fastidious and anaerobic organisms were not used and this may have influenced the findings. In addition, the majority of bacteria were identified to genus level. Species detection by ribotyping and API testing were not performed. The use of culture methods limits the detection of viable but non-culturable organisms (VBNC). Alternative methods of molecular testing using 16s ribotyping were considered. However this method limits quantification of bacteria, which was possible using culture techniques. In addition, molecular methods can also produce a plethora of organisms with no information on their pathogeneric properties or whether they are alive.

7.2 Urinary ATP as a marker of infection

There is now much evidence to show that the current tests used to exclude UTI are not adequate. The problems affect the culture methods used to exclude UTI and the surrogate tests; dipsticks and even microscopy for pyuria. The current gold standard used in most UK laboratories continues to be microbial culture of a single known urinary pathogen of more then 10^5 cfu ml^{-1}. This threshold first purposed by Kass (1957) for women with clinical pyelonephritis, suffers from spectrum bias and should not be used to screen all patients with LUTS (38, 40). In addition, there is now much evidence for polymicrobial infection as opposed to infection from a single predominate organism (41, 150, 184). It is now well recognised that the threshold used to define acute infection of the lower urinary tract is much too high (46, 48, 180, 185, 186). Hence use of these tests in patients with chronic infection needs to be interpreted with caution (47). Very few studies have looked at the use of dipsticks and culture in patients with chronic LUTS (187-189). There is much work need to explore alternative methods to screen for infection in patients with LUTS. ATP has been proposed as a potential clinical marker for acute and chronic urinary tract disease (37, 124, 190). Optimum clinical sampling methods were reviewed along with whether urinary ATP explains the symptoms and markers of infection. This study finds that urinary ATP showed little concordance with the clinical consequences of urinary infection in chronic LUTS, and therefore does not show promise for future development of a diagnostic test for this particular disease. Urinary ATP was found lacking and did not improve on fresh microscopy of urine to screen for infection. There is need to explore other potential markers that can be used to screen for infection in LUTS patients, particularly applicable to those that have lower levels of pyuria, where significant disease may currently be

overlooked. Abundant urinary ATP is certainly evident in the presence of significant disease amongst patients with LUTS and these data do encourage continued interest in the pharmacology and pathophysiology of purinergic functions in the bladder.

7.3 The cytokine response in OAB

The proposed relationship between urinary infection and the generation of OAB symptoms has been suggested from cross-sectional studies. The literature has a range of potential biomarkers for OAB but none have been reviewed in relation to chronic infection and the generation of OAB symptoms. This is the first study to explore urinary IL6, CXCL 8, Lactoferrin, Tamm-Horsfall Protein (THP), Monocyte Chemoatractant Protein-1 (MCP) and Nerve Growth Factor (NGF) in patients with OAB symptoms, as compared to asymptomatic controls, in relation to other markers of inflammation. Urinary IL6 and Lactoferrin showed a significant difference between patients and controls. Levels of urinary IL6 and Lactoferrin varied in relation to pyuria and total microbial growth suggesting a correlation between inflammation and bacterial infection in a symptomatic population. The methods used in this work were hypothesis driven. Others have conducted similar work but used micro-array methods to screen very large numbers of potential markers. Such an approach is susceptible to numerous Type 2 errors because probability favours the occurrence of chance correlations unrelated to causative associations. This problem is best avoided by adopting a hypothesis-based approach that scrutinises a limited number of plausible candidates. I selected this panel of cytokines on my understanding of the pathophysiology being studied and used conventional empirical science to prove or refute a hypothesis.

7.4 Urinary infection and inflammation in OAB

The current diagnostic tests used to screen for infection are clearly inadequate and urinary infection is being missed through use of fixed diagnostic thresholds. Using new culture techniques looking at the urinary sediment, there was a distinct difference found in the urinary ecology between patients with OAB symptoms and matched controls. This was supported by evidence of urothelial inflammation through examination of pyuria, epithelial cell shedding and urinary cytokines. Whilst quantitative differences in bacterial growth were observed, there was additionally a difference in the organisms found in those with symptoms; with recognised uropathogens including *E. coli* and *Enterococcus spp*. isolated more frequently. Routine urine culture did not detect these differences. These ecological findings were associated with evidence of increased urothelial inflammation amongst patients, implying that these microbes might be more than harmless contaminants.

The proposed relationship between urinary infection and the generation of OAB symptoms was studied. An observational cohort study was undertaken with patients and controls matched for key demographic characteristics and monitored at twelve study visits over a one-year period. Pyuria and epithelial cell shedding was associated with increased bacterial colony counts and increasing pyuria was associated with a rise in bacterial load. Increasing pyuria was associated with elevated levels of urinary ATP, Lactoferrin and IL6, indicative of urothelial distress and inflammation, although the strength of these relationships varied.

Increasing pyuria was associated with deterioration in total symptoms, epithelial cell shedding and microbial growth. These relationships were independent of routine culture status. These controlled, prospective data are the first to demonstrate a

significant association between bacterial infection, urothelial inflammation and the presence of OAB symptoms. These data suggest that the treatment of presumed bacterial infection in the presence of pyuria might confer significant symptomatic benefit.

7.5 Further work

The approach to the diagnosis of UTI needs to be revised and the disease should no longer be viewed as a categorical dichotomy of positive or negative. Rather than pursuing new diagnostics, to define the presence or absence of the condition, we might achieve greater success by applying Bayesian models and think in terms of probabilities when judging our approach to the treatment of patients. Bayesian methods feed on broad knowledge and a deeper understanding of the interaction between bacteria and host. Biology always forms continua and is inimical to categorisation. Nowadays we have the technology needed to deal with numerous probability distributions that are relevant to a particular pathology. It is the norm for data to be entered onto computer systems. There is no reason why probability calculations that incorporate all of the available data so that richer analysis of diagnoses and treatment decisions can be achieved.

Studies to explore the prevalence of atypical bacterial infection in these patients would compliment this work. The longitudinal data from this study indicate that patients with OAB symptoms demonstrate evidence of bacterial infection and hence intervention studies looking at the effect of treatment of this would be important.

7.6 Conclusions

The hypothesis that 'Bacterial infection of the lower urinary tract goes undetected by routine diagnostic testing and contributes to the generation of lower urinary tract symptoms in patients with symptoms of overactive bladder' was successfully tested and the results support this hypothesis.

The aims of the study were:

1. To explore the urinary inflammatory response in patients with symptoms of overactive bladder.

Bacterial infection of the lower urinary tract goes undetected by routine diagnostic testing and there is evidence of urinary infection and inflammation in patients with symptoms of OAB that is significantly different to asymptomatic controls.

2. To determine the relationship between urothelial inflammation, manifested by pyuria, epithelial shedding and microbial growth, and symptom variation in patients with symptoms of overactive bladder.

Patients with OAB show a difference in pyuria and epithelial cell shedding when compared to controls. Patients with OAB show significantly higher microbial growth when compared to controls. When explored further by qualitative analysis the bacterial ecology in patients with OAB is distinctly different to controls.

3. To determine the urinary cytokine and inflammatory response in relation to symptom exacerbation in OAB.

Urinary IL6 and Lactoferrin were found to be significantly higher in patients with OAB symptoms as compared to controls. These markers varied in accordance with pyuria, microbial growth and epithelial cell shedding. These markers may provide promise in exploring infection and inflammation in patients with OAB symptoms.

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9 Appendices

9.1 Study Information sheet

The Whittington Hospital NHS
NHS Trust

Department of Medicine
1st Floor, Clerkenwell Building
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London N19 5LW

Participant Information Sheet - Patient

Short title: Immunology of Cystitis and OAB

A blinded observational cohort study of the immunological changes associated with chronic cystitis and overactive bladder symptoms in women.

Version 2.0, 21st February 2011

We would like to invite you to take part in our research study. Before you decide we should like you to understand why the research is being done and what it would involve for you. One of our team will go through the information sheet with you and answer any questions you have. We suggest that this should take about 20 minutes. Talk to others about the study if you wish.

Part 1 tells you the purpose of this study and what will happen to you if you take part.

Part 2 gives you more detailed information about the conduct of the study.

Ask us if there is anything that is not clear. Take time to decide whether or not you wish to take part.

Participant Information Sheet – Part 1

What is the purpose of the project?

Many people suffer with Overactive Bladder, causing symptoms such as needing to rush to the toilet, passing urine frequently (more than 8 times per day and once at night) and urinary incontinence (leaking from the bladder). Additionally there may be problems with bladder emptying and with recurrent urinary

infection. We recognise that the symptoms are very unpleasant and that the urine infections are particularly disruptive.

We have been working with patients who suffer from bladder troubles for many years. We have discovered that many of those people with overactive bladder symptoms and those with recurrent urine infection demonstrate a previously unrecognised inflammatory reaction in the urine that is evident provided that the urine is examined very fresh by a light microscope. When these specimens are sent to the ordinary laboratory more than half are reported as not showing infection, termed culture negative. We have developed a better method for analysing the samples that uses very sensitive microbiological methods and found that we can identify bacterial infection in over 80%.

We have some evidence that our affected patients are not suffering from recurrent urine infection but from the same chronic infection, going on for months or years, which from time to time becomes acutely worse for periods. What is more, we believe that the bacteria are living inside the cells of the bladder where they are protected from immune and antibiotic attack so that treatment has to use methods that are different to those used to treat ordinary urinary infection.

We seek to find out whether this is definitely the case in patients with overactive bladder. We want to know whether such infections may be adding to the problems that affect the bladder. We also want to find out whether treating people with antibiotics that we know the bacteria to be sensitive to makes a difference to a person's symptoms. We are also interested in studying newer and more sensitive markers of infection.

Why have I been invited?

We hope to include 90 participants in this study which include a mixture of people with overactive bladder and others with no bladder symptoms. You have been chosen as you:

- Are female
- Have symptoms of overactive bladder
- May or may not have signs of infection in the urine

Do I have to take part?

It is up to you if you decide whether or not to take part. It is up to you to decide to join the study. We will describe the study and go through this information sheet. If you agree to take part, we will then ask you to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason. This will not affect the standard of care you receive.

What will happen to me if I take part?

If you decide to take part in the project the research clinician will arrange to see you. You will be asked some questions about your bladder symptoms. Your answers will be recorded in your confidential research record and stored on a computer. This will take about 30 minutes. The computer record is closely guarded by the NHS security system so there is no unauthorised access to your record.

A urine specimen will be taken by the midstream method. Part of it will be sent to the laboratory to check for any bacteria in your urine and another portion will be

looked at under the microscope to detect small 'white blood cells,' which can signify an infection in the urine. Sometimes, tests that look for bacteria in the laboratory can come back as negative and looking for white blood cells under the microscope is another way to look for infection that may otherwise have been missed. We should like to record the results of these in your research recorded. The urine will also be used to examine the inflammatory reaction that is occurring in your bladder. A blood sample will also be taken and a skin allergy test performed. These will be required only once throughout the study.

Once we have entered you into the study we should like to review you once a month for a year when we shall check your symptoms and obtain a urine sample. During this time you will be treated for your symptoms and if there are any signs of infection in the urine you will be offered treatment with antibiotics. As the bacteria tend to hide within bladder cells you may need to be on antibiotics for 6-9 months and will be closely monitored. This is standard treatment for patients in our clinic.

A key inconvenience is that you will need to visit the centre once each month. You will, however, be seen outside of the normal clinic and at a time convenient to you.

Blood Test

All participants will need to have a blood test at their initial visit. This will involve the researcher taking a small blood sample of 20mls from a vein in your arm. This may cause some mild bruising of the area which will usually resolve in a few days.

Allergy Skin Test

Candida skin tests will be performed on all participants at visit 2. This is a test commonly used to analyse the participants immune response. This will involve having a small injection on the inner side of your lower arm. The injection site will be marked with indelible ink. 48 hours later you will need to come back and the site will be checked for signs of redness and this will be measured. Two small skin biopsies (3mm each in size) will be taken under local anaesthetic. The local anaesthetic numbs the small area of skin from where the biopsies are taken so that it is not painful. The numbness can last upto 1 hour. This test will be performed by the Researcher.

Flexible Cystoscopy

If you are in the group patients with overactive bladder, we shall ask you to undergo a flexible cystoscopy and biopsy at some time but only once. This will allow us to examine your bladder more thoroughly and ascertain the degree of inflammation that might be affecting your bladder. If you are in the control group you will NOT need to have this procedure done.

Who will perform my procedure?

A doctor trained in urology, surgery of the bladder, will perform the procedure.

What is the procedure?

A doctor will insert an instrument called a cystoscope into your bladder via the water pipe (urethra). The instrument is a very fine and soft telescopic lens and attached to this is a light source and some sterile water to fill the bladder so that the lining can be inspected. A local anaesthetic gel is used to numb and lubricate the urethra to make the passage into the bladder as comfortable as possible.

Some patients may experience slight discomfort during the procedure but the majority do not. If you do feel uncomfortable at any time you should inform the doctor performing the examination immediately.

Once the instrument is in place, the examination will take only a few minutes to complete.

Once the inspection is completed, the instrument will be removed, and you will be informed of the findings and the need for any further management.

A nurse will remain with you whilst the treatment is taking place and will explain anything you do not understand.

Are there any serious or frequently occurring risks?

There are potential complications with any procedure. Although these are rare, it is important that you are aware of them and have the opportunity to discuss them with your doctor.

Common
Mild burning or bleeding on passing urine for a short period after the cystoscopy.

Occasional
Infection of the bladder requiring antibiotics. We shall be giving you a course of antibiotic treatment lasting one day at the time of the procedure

Rare
Delayed bleeding requiring removal of clots or further surgery. Injury to the urethra causing delayed scar formation. Difficulties in passing urine after the procedure requiring temporary insertion of a catheter.

What does the bladder biopsy entail?

When you have the cystoscopy the doctor will take a very small sample of bladder tissue (about 1-2mm) in addition to any that may be needed as part of your normal clinical care. It feels like a sharp scratch in your bladder, which is uncomfortable. The biopsy site from your bladder may bleed but it will stop bleeding by itself. There is no need to do anything to the area of the biopsy site to stop bleeding, as the sample of bladder tissue taken is very small and shallow. Whilst bladder biopsies are a common feature of normal patient care, this biopsy would be over and above those involved in normal care.

Expenses and Payments

We shall be able to reimburse you for travel expenses incurred from attending study assessments and for additional food and drink arising from this travel. We should be grateful for receipts describing the expenses for our auditors. We are not in a position to pay you for participation in the study.

Other studies

It would not be advisable for you to participate in this study if you are already a subject in another trial.

Pregnancy

It would not be advisable for you to participate in this study if you are pregnant or planning to become pregnant during the study period. Unless you are post-menopausal and not had a period for more than 2 years, a urine pregnancy test will be preformed on all participants at the beginning of the study and before the cystoscopy.

What will I have to do?

You will have to:

1. Give your consent
2. Attend the hospital for your scheduled visits once each month
3. Provide urine specimens at your visits.
4. If you are in agreement, undergo a cystoscopy and bladder biopsy

Study schedule

Day Identification and visit	**Actions**
Pre-study contact	Informal recruitment process and participant sent information sheet
Contact at around day -14	Participant contacted to set date of screening visit
Day 0 Visit 1	1. Informed consent 2. Urine Pregnancy test 3. Participant completes 3 questionnaires 4. Midstream urine (MSU) sample obtained 5. Urine stored for analysis 6. Blood sample taken
Visit 2 at 1 month	1. Participant completes 3 questionnaires 2. MSU sample obtained 3. Urine stored for analysis 4. At Visit 2 a skin test will be preformed
Visit 3 at 1 month and 2 days	Skin test reviewed 2 x 3mm skin biopsies taken
Visits 4 – 14 (every month)	1. Participant completes 3 questionnaires 2. MSU sample obtained 3. Urine stored for analysis
Additional visits every two to three days over the period of acute worsening of symptoms	1. Participant completes 3 questionnaires 2. MSU sample obtained 3. Urine stored for analysis

Biopsy Visit	1. Participant completes 3 questionnaires 2. Urine Pregnancy test 3. MSU sample obtained 4. Urine stored for analysis 5. Flexible cystoscopy and bladder biopsy

What about my current treatments for other conditions?

You will be able to continue all of your normal treatments and these will not be affected by this study.

What are the alternatives to participating in the study?

It is entirely your decision if you wish to take part in the study and it will not affect your future care if you do not wish to do so. If you do not wish to take part you will have your assessment in the usual way and your condition will be managed as is done routinely by your consultant.

What are the possible disadvantages and risks of taking part?

You will be seen more often than would be normal in this clinic. However, you will be seen outside of the clinic and will not have to wait as in an ordinary clinic. We shall do our best to book you in line with your convenience. We shall ask to perform a cystoscopy and bladder biopsy and that is explained in a separate part of this information package.

If you ever experience a sudden worsening of your bladder symptoms we would like to see you immediately, on the same day, so as to get these resolved quickly. This could be inconvenient but it could also be very advantageous to you.

What are the possible benefits of taking part?

There may not be any benefit, however, you will have access to unusually close monitoring of your bladder symptoms.

What happens when the research study stops?

We shall be pleased to continue with your normal clinical care in the usual ways.

What if there is a problem?

Any complaint about the way you have been dealt with during the trial or any possible harm you might suffer will be addressed. The detailed information concerning this is given in Part 2 of this information sheet. If you have any concerns or complaints you should contact your study doctor in the first instance.

Will my taking part in the study be kept confidential?

Yes. We will follow ethical and legal practice and all information about you will be handled in confidence. The details are included in Part 2.

Contact Details

Your Doctor
Name **Professor James Malone-Lee** Tel. Number: **020 7288 5589**

Your Research Fellow
Name **Dr Kiren Gill** Tel. Number: **020 7288 3009**

This completes Part 1 of the Information Sheet.

If the information in Part 1 has interested you and you are considering participation, please read the additional information in Part 2 before making any decision.

Participant Information Sheet – Part 2

What if new information becomes available?

Sometimes during the course of a research project, new information becomes available about the disease area that is being studied. If this happens, your research doctor or nurse will tell you about it, and discuss with you whether you want to continue in the study.

If you decide to withdraw your research doctor will make arrangements for your care to continue.

If you decide to continue you will be asked to sign an updated consent form. Also, on receiving new information your research doctor might consider it to be in your best interests to withdraw you from the study. He/she will explain the reasons and arrange for your care to continue.

If the study is stopped for any other reason, you will be told why and your continuing care will be arranged.

What will happen if I don't want to carry on with the study?

You are free to withdraw from the study at any time before and after signing the consent form without needing to give any explanations. The study may be ended at any time with or without your consent.

Should you wish to leave the study we shall offer you a general check-up and the option of continued outpatient follow-up.

Unless you wish otherwise, the data collected from you up to the point at which you leave the study will be used in the analysis.

What if there is a problem?

Every care will be taken to ensure your safety during the course of the study. If you have a concern about any aspect of this study, you should ask to speak with the researchers who will do their best to answer your questions. Please contact **Dr Kiren Gill or Professor Malone-Lee** in the first instance

Harm

In the event that something does go wrong and you are harmed as a result of taking part in the approved research study UCL, the Research Sponsor, has indemnity (insurance) arrangements in place for non-negligent harm. If you are harmed and this is due to someone's negligence then you may have grounds for legal action for compensation against the Trust but you may have to pay your legal costs.

Every care will be taken in the course of this clinical trial. However in the unlikely event that you are injured by taking part, compensation may be available.

If you suspect that the injury is the result of the Sponsor's (University College London) or the hospital's negligence then you may be able to claim compensation. After discussing with your study doctor, please make the claim in writing to Professor Malone-Lee who is the Chief Investigator for the clinical trial and is based at Department of Medicine, Clerkenwell Building, Highgate Hill, London N19 5LW. The Chief Investigator will then pass the claim to the Sponsor's Insurers, via the Sponsor's office. You may have to bear the costs of the legal action initially, and you should consult a lawyer about this.

Participants may also be able to claim compensation for injury caused by participation in this clinical trial without the need to prove negligence on the part of University College London or another party. You should discuss this possibility with your study doctor in the same way as above.

Complaints

If you have any questions about your rights as a research subject or have a complaint about the way in which the study has been carried out, please contact: Ms Senga Steel, Assistant Director of Nursing, Whittington Hospital NHS Trust, Magdala Avenue, London N19 5NF; Tel 020 7288 3405

If you remain unhappy and wish to complain formally, you can do this through the NHS Complaints Procedure. Details can be obtained from the hospital or from the Department of Health website: http://www.dh.gov.uk. You may obtain the necessary guidance from the hospital Patient Advice and Liaison Service (PALS), Whittington Hospital, Tel 020 7288 5956 or 020 7288 5957

Will my taking part in this study be kept confidential?

If you consent to take part in this study, the records obtained while you are in this study as well as related health records will remain strictly confidential at all times. The information will be held securely on paper and electronically at the hospital site managing this research under the provisions of the 1998 Data Protection Act. Your name will not be passed to anyone else outside the research team or the Sponsor (UCL), who is

not involved in the trial. You will be allocated a trial number, which will be used as a code to identify you on all trial forms. Any information about you which leaves the hospital will have your name and address removed so that you cannot be recognised (if it is applicable to your research).

Your records will be available to people authorised to work on the trial but may also need to be made available to people authorised by the Sponsor, which is the organisation responsible for ensuring that the study is carried out correctly. By signing the consent form you agree to this access for the current study and any further research that may be conducted in relation to it, even if you withdraw from the current study.

If you withdraw consent from further study treatment, unless you object, your data and samples will remain on file and will be included in the final study analysis.

In line with the regulations, at the end of the study your data will be securely archived for a minimum of 15 years. Arrangements for confidential destruction will then be made.

Will my GP be informed of my involvement?

With your permission, your GP, and other doctors who may be treating you, will be notified that you are taking part in this study.

What will happen to any samples I give?

A urine specimen will be taken and part of it will be sent to the hospital laboratory to check for any bacteria in your urine and another portion will be looked at under the microscope to detect small 'white blood cells,' which can signify an infection in the urine. Two bladder biopsies will be taken at the time of your cystoscopy. One will be sent to the hospital laboratory for analysis and the other specimen will be analysed by the researcher. Samples will not be labelled with information that can directly identify you, and will only be labelled with the study number that has been allocated to you. A portion of the urine samples will be frozen and stored for further analysis of inflammation chemicals and bacterial proteins. We think that it may play an important role in the development of over-active bladder symptoms. The anonymised samples will be stored in a monitored freezer in a secure laboratory in the Department of Medicine, Clerkenwell Building. The samples will be analysed by researchers within the Division of Medicine at UCL. Once analysed the urine sample will be disposed of as per UCL regulations.

Will any genetic tests be done?

No genetic tests will be done.

What will happen to the results of the research?

The results of the study may be published in scientific journals so that other researchers working on improving treatments for people with troublesome bladder symptoms and incontinence will benefit from our knowledge. You will not be identified of any publications and we shall provide you in any case with a report of the results of the trial.

Who is organising and funding the research?

This study is organised by the Department of Medicine at the Whittington Hospital. We are currently making applications for research funds to support this programme and are funded this work from our own resources at this time. The study is sponsored by University College London.

Who has reviewed the study?

All research in the NHS is looked at by independent group of people, called a Research Ethics Committee, to protect your interests. This study has been reviewed and given favourable opinion by East Central London Research Ethics Committee (1).

Further information and contact details

You are encouraged to ask any questions you wish, before, during or after your treatment. If you have any questions about the study, please speak to your study nurse or doctor, who will be able to provide you with up to date information about the drug(s)/procedure(s) involved. If you wish to read the research on which this study is based, please ask your study nurse or doctor. If you require any further information or have any concerns while taking part in the study please contact one of the following people:

Your Doctor
Name **Professor James Malone-Lee** Tel. Number: **020 7288 5589**

Your Research Fellow
Name **Dr Kiren Gill** Tel. Number: **020 7288 3009**

If you decide you would like to take part then please read and sign the consent form. You will be given a copy of this information sheet and the consent form to keep. A copy of the consent form will be filed in your patient notes, one will be filed with the study records and one may be sent to the Research Sponsor.

You can have more time to think this over if you are at all unsure.

Thank you for taking the time to read this information sheet and to consider this study.

9.2 Study Consent Form

Whittington Health NHS

Department of Medicine
1st Floor, Clerkenwell Building
Archway Campus
Highgate Hill
London N19 5LW

CONSENT FORM

A blinded observational cohort study of the immunological changes associated with Chronic Cystitis and Overactive Bladder symptoms in women

Study Number: Participant Identification Number for this trial:

Name of Researcher:

1. I confirm that I have read and understand the information sheet dated..... (version.....) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected. ☐

3. I understand that relevant sections of my medical notes and data collected during the study, may be looked at by individuals from the sponsor of the trial (University College London), from regulatory authorities or from the NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records. ☐

4. I agree to my GP being informed of my participation in the study. ☐

5. I agree to take part in the above study. ☐

Name of Patient	Date	Signature
Name of Person taking consent	Date	Signature

Name of Chief Investigator	Date	Signature
(if different to the person taking consent)		

When completed: 1 for participant; 1 (original) for researcher site file; 1 to be kept in medical notes.

9.3 Ethics Approval

NHS

National Research Ethics Service

East Central London REC 1
South House, Block A
Royal Free Hospital
Pond Street
London
NW3 2QG

Telephone: 020 7794 0552

Professor James Malone-Lee
Department of Medicine
Centre for Clinical Science, Technology & Geriatrics
Clerkenwell Building
Highgate Hill, London
N19 5LW

17 February 2011

Dear Professor Malone-Lee

Study Title: **A blinded observational cohort study of the immunological changes associated with chronic cystitis and overactive bladder symptoms in women**

REC reference number: **11/H0721/7**

The Research Ethics Committee reviewed the above application at the meeting held on 12 January 2011. Thank you for attending with Dr Kiren Gill to discuss the study.

After the Committee's initial deliberations you were invited to join the meeting to clarify some issues. You kindly clarified the following issues with the Committee:

i. The Committee asked you if the antibiotic treatment was longer that standard practice. You explained the difference between non-inflamed and inflamed Overactive Bladder disease and that all patients were limited to 6-9 months treatment. The Committee said that this should be made clear in the Information Sheet.
ii. The Committee informed you that the Information Sheet should state what size the skin biopsy would be and how much blood would be taken.
iii. The Committee asked whether the different groups would be age-matched. You confirmed that they would be.
iv. The Committee informed you that there were several typing errors in the Information Sheet and the language need to be simplified in lay terms.
v. The Committee informed you that the Candida skin test should be explained.
vi. The Committee stated that there should be Information Sheets for all three groups and should be written in lay language

Ethical opinion

The members of the Committee present gave a favourable ethical opinion of the above research on the basis described in the application form, protocol and supporting documentation, subject to the conditions specified below:

Ethical review of research sites

The favourable opinion applies to all NHS sites taking part in the study, subject to

management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Information Sheets:

1. There should be an Information Sheet and Consent Form for each group participating. There is no need to ask for access to medical notes for controls.
2. After the introduction, 'Part 1' should be added before the section 'What is the purpose of the project?'
3. The last sentence of the first paragraph in the section, 'What is the purpose of the project? ' does not make sense. The symptoms have not been fully explained.
4. In the section, 'Why have I been invited?' the descriptions should be bullet pointed and there should be a section stating that the participants are women. This section will vary in each Information Sheet.
5. In the section, 'What will happen to me if I take part?' the wording at the top of page 3 should read, '...in your confidential research record.'
6. In the same paragraph, the wording should read, 'A blood sample will also be taken and **a skin** allergy test performed. These will be required only once throughout the study'
7. The Participants should also be informed of how much blood will be taken and that this may cause bruising.
8. There should be an explanation that the Candida antigen skin test will be done to analyse the participant's immune response, as some women may be alarmed by this test. The Information Sheet should also mention how large the biopsies will be and what the effects of the local anaesthetic could be.
9. In the section, 'What is the procedure?' the third line should read, '...the instrument **is** a telescopic lens.'
10. In the section, 'Are there any serious or frequently occurring risks?' the word 'Operation' should be changed to 'Cystoscopy.'
11. In the section, 'Expenses and Payments' the word 'trail' should be changed and 'sustenance' should be changed to 'refreshments' or 'food and drink.'
12. In the section on Pregnancy, there should be more detail on who will need to undergo a pregnancy test and whether this is necessary for post-menopausal women.
13. The Committee thought that the Study schedule was too complicated and would not be easily understood by participants. The terms MSU and ATP were not explained. The box describing the visits seemed to have an extra 'day' in each line, which should be removed. A simplified flow chart showing the visits and procedures that would be performed would be much clearer.
14. In the section 'What about my current treatment for other conditions?' the word 'effected' should be 'affected.'
15. In the section, 'What are the possible disadvantages and risks of taking part?' the word 'exacerbation' should be simplified.
16. In the section 'What will happen to any samples I give?' the order of clauses in sentence three should be rearranged to show that one sample will go to the hospital

laboratory and the other specimen will be used for research. This can then be followed by the information that these research samples will be unidentifiable.

17. In the section 'Who is organising and funding the research?' the word 'tie' should be 'time.'
18. The participant should be informed of the use of antibiotics in the study such as how long they may expect to take antibiotics and whether this is standard treatment, etc.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

For NHS research sites only, management permission for research ("R&D approval") should be obtained from the relevant care organisation(s) in accordance with NHS research governance arrangements. Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk.

Where the only involvement of the NHS organisation is as a Participant Identification Centre (PIC), management permission for research is not required but the R&D office should be notified of the study and agree to the organisation's involvement. Guidance on procedures for PICs is available in IRAS. Further advice should be sought from the R&D office where necessary.

Sponsors are not required to notify the Committee of approvals from host organisations.

It is responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

You should notify the REC in writing once all conditions have been met (except for site approvals from host organisations) and provide copies of any revised documentation with updated version numbers.

Approved documents

The documents reviewed and approved at the meeting were:

Document	*Version*	*Date*
REC application		09 December 2010
Protocol	1	06 December 2010
GP/Consultant Information Sheets	1	06 December 2010
Participant Information Sheet	1	06 December 2010
Investigator CV		05 August 2010
Participant Consent Form	1	06 December 2010
Covering Letter		09 December 2010
Evidence of insurance or indemnity		06 December 2010
Questionnaire: Validated		
CV Dr Kiren Gill		06 November 2010

Membership of the Committee

The members of the Ethics Committee who were present at the meeting are listed on the attached sheet.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Now that you have completed the application process please visit the National Research Ethics Service website > After Review

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.

The attached document "After ethical review – guidance for researchers" gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Progress and safety reports
- Notifying the end of the study

The NRES website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

We would also like to inform you that we consult regularly with stakeholders to improve our service. If you would like to join our Reference Group please email referencegroup@nres.npsa.nhs.uk.

11/H0721/7 **Please quote this number on all correspondence**

With the Committee's best wishes for the success of this project

Yours sincerely

[redacted]

Dr David Slovick
Chair

Enclosures: *List of names and professions of members who were present at the meeting and those who submitted written comments*
"After ethical review – guidance for researchers

Copy to: *Mr David Wilson, R&D office*

9.4 ICIQ FLUTS Questionnaire

ICIQ FLUTS Long Form August 2004
Copyright © "ICIQ Group"
Hybridised by James Malone-Lee
31/12/2010

ICIQ FLUTS Long Form
Whittington Urgency Score
Whittington Bladder Pain Score

This questionnaire combines with ICIQ-FLUTS Long Form with the Whittington Urgency Score and the Whittington Bladder Pain Score. This long form of the questionnaire contains important questions about voiding ability that are germane to the occurrence of urine infection

Patient Number_____ Patient Initials_____
Date_____

Urinary Symptoms

We should be grateful if you would answer the following questions, thinking about how you have been, on average, over the **PAST FOUR WEEKS.**

1. **Please write your date of birth**_____

2A. **How often do you pass urine during the day**?

One to six times	0	
Seven to eight times	1	
Nine to ten times	2	
Eleven to twelve times	3	
Thirteen times or more times	4	

2B. **How much does this bother you?**
(Please ring a number between 0 (not at all) and 10 (a great deal)

0 1 2 3 4 5 6 7 8 9 10
Not at all **A great deal**

3A. **During the night, how many times do you have to get up to urinate, on average**?

None	0	
One	1	
Two	2	
Three	3	
Four or more	4	

3B. **How much does this bother you?**
(Please ring a number between 0 (not at all) and 10 (a great deal)

0 1 2 3 4 5 6 7 8 9 10
Not at all **A great deal**

4A. **Do you have a sudden need to rush to the toilet to urinate**?

Never	0	
Occasionally	1	
Sometimes	2	
Most of the time	3	
All of the time	4	

4B. **How much does this bother you?**
(Please ring a number between 0 (not at all) and 10 (a great deal)

0 1 2 3 4 5 6 7 8 9 10
Not at all **A great deal**

4C. **If you <u>do</u> experience urgency, a sudden need to rush to the toilet to urinate,…...**

	Tick if Yes
Does cold weather make matters worse?	
Does the sound of running water cause urgency?	
Does putting a key in your door or nearing home cause urgency?	
Does getting out of bed in the morning cause urgency	
Does fatigue or anxiety make mattes worse?	

5A. **Does urine leak before you can get to the toilet**?

Never	0	
Occasionally	1	
Sometimes	2	
Most of the time	3	
All of the time	4	

5B. **How much does this bother you?**
(Please ring a number between 0 (not at all) and 10 (a great deal)

0 1 2 3 4 5 6 7 8 9 10
Not at all **A great deal**

5C. **If you <u>do</u> experience urge incontinence, a urine leak before you can get to the toilet,…...**

	Tick if Yes
Does the sound of running water cause incontinence?	
Does putting a key in your door or nearing home cause incontinence?	
Does getting out of bed in the morning cause incontinence?	

6A. **Do you have pain in your bladder**?

Never	0	
Occasionally	1	
Sometimes	2	
Most of the time	3	
All of the time	4	

6B. **How much does this bother you?**
(Please ring a number between 0 (not at all) and 10 (a great deal)

0 1 2 3 4 5 6 7 8 9 10
Not at all **A great deal**

6C. **If you <u>do</u> experience bladder pain,…...**

		Tick if Yes
Do you experience discomfort or pain on bladder filling?		
Is there pain or discomfort in or over the pubic area?		
	Is this pain or discomfort relieved completely by passing urine?	
	Is this pain or discomfort partially relieved by passing urine?	
	Is this pain or discomfort unrelieved by passing urine?	
Is there bladder pain or discomfort whilst passing urine?		
Is there pain or discomfort after passing urine?		
Is there pain in your back over your kidneys?		
Is there pain referred to or felt in the genitals?		
Is there pain on the left or right side of the lower abdomen?		
Is there general abdominal pain?		
Is there pain radiating down your legs?		

7A. **How often do you leak urine**?

Never	0	
Once or less per week	1	
Two or three times per week	2	
Once per day	3	
Several times per day	4	

7B. **How much does this bother you?**
(Please ring a number between 0 (not at all) and 10 (a great deal)

0 1 2 3 4 5 6 7 8 9 10
Not at all **A great deal**

8A. **Does urine leak when you are physically active, exert yourself, cough or sneeze**?

Never	0	
Occasionally	1	
Sometimes	2	
Most of the time	3	
All of the time	4	

8B. **How much does this bother you?**
(Please ring a number between 0 (not at all) and 10 (a great deal)

0 1 2 3 4 5 6 7 8 9 10
Not at all **A great deal**

9A **Do you ever leak for no obvious reason and without feeling that you want to go?**

Never	0	
Occasionally	1	
Sometimes	2	
Most of the time	3	
All of the time	4	

9B. **How much does this bother you?**
(Please ring a number between 0 (not at all) and 10 (a great deal)

0 1 2 3 4 5 6 7 8 9 10
Not at all **A great deal**

10 **How much urinary leakage occurs?**

No leakage	0	
Drops/pants damp	1	
Dribble/pants wet	2	
Floods, soaking through to outer clothing	3	
Floods running down legs onto the floor	4	

11A. **Is there a delay before you cant start to urinate**?

Never	0	
Occasionally	1	
Sometimes	2	
Most of the time	3	
All of the time	4	

11B. **How much does this bother you?**
(Please ring a number between 0 (not at all) and 10 (a great deal)

0 1 2 3 4 5 6 7 8 9 10
Not at all **A great deal**

12A. **Do you have to strain to urinate**?

Never	0	
Occasionally	1	
Sometimes	2	
Most of the time	3	
All of the time	4	

12B. **How much does this bother you?**
(Please ring a number between 0 (not at all) and 10 (a great deal)

0 1 2 3 4 5 6 7 8 9 10
Not at all **A great deal**

13A. **Do you stop and start more than once while you urinate**?

Never	0	

Occasionally	1	
Sometimes	2	
Most of the time	3	
All of the time	4	

13B. **How much does this bother you?**
(Please ring a number between 0 (not at all) and 10 (a great deal)

0 1 2 3 4 5 6 7 8 9 10
Not at all **A great deal**

14A. **Do you leak urine when you are asleep**?

Never	0	
Occasionally	1	
Sometimes	2	
Most of the time	3	
All of the time	4	

14B. **How much does this bother you?**
(Please ring a number between 0 (not at all) and 10 (a great deal)

0 1 2 3 4 5 6 7 8 9 10
Not at all **A great deal**

15A. **Would you say that the strength of you urinary stream is……..**?

Not reduced	0	
Reduced a little	1	
Quite reduced	2	
Reduced a great deal	3	
No steam	4	

15B. **How much does this bother you?**
(Please ring a number between 0 (not at all) and 10 (a great deal)

0 1 2 3 4 5 6 7 8 9 10
Not at all **A great deal**

16. **Have you ever blocked up completely so that you could not urinate at all and had to have a catheter to drain the bladder**?

No	0	
Yes, once	1	
Yes, twice	2	
Yes, more than twice	3	

17A. **Do you have a burning feeling when you urinate**?

Never	0	
Occasionally	1	
Sometimes	2	
Most of the time	3	
All of the time	4	

17B. **How much does this bother you?**
(Please ring a number between 0 (not at all) and 10 (a great deal)

0 1 2 3 4 5 6 7 8 9 10
Not at all **A great deal**

18A. **How often do you feel that your bladder has not emptied properly after you have urinated**?

Never	0	
Occasionally	1	
Sometimes	2	
Most of the time	3	
All of the time	4	

18B. **How much does this bother you?**
(Please ring a number between 0 (not at all) and 10 (a great deal)

0 1 2 3 4 5 6 7 8 9 10
Not at all **A great deal**

19. **Can you stop the flow of urine if you try while you are urinating**?

Yes, easily	0	
Yes, with difficulty	1	
No, cannot stop it flowing	2	

9.5 Whittington Urgency score

Whittington Urgency Score

Please enter date of completion: **dd-mmm-yy**

We should be grateful if you would answer the following questions, thinking about how you are at the moment. Please put a circle around your response for each question.

Do you experience urgency? *That is having to hurry in order to pass urine*	None	Some	Much
Do you experience urge incontinence? *That is hurrying to pass urine and not making it in time*	None	Some	Much
Does cold weather make your bladder symptoms worse?	None	Some	Much
Do you find that running water from a tap causes urinary urgency?	None	Some	Much
Do you find that running water from a tap causes incontinence?	None	Some	Much
Do you find that putting a key in the front door when returning home causes urinary urgency?	None	Some	Much
Do you find that putting a key in the front door when returning home causes urinary incontinence?	None	Some	Much
Do you find that on getting up from bed in the morning you experience urgency?	None	Some	Much
Do you find that on getting up from bed in the morning you experience urge incontinence?	None	Some	Much
Does anxiety or fatigue make your symptoms worse?	None	Some	Much

Score 0 for none
Score 1 for some
Score 2 for much

Total score	

9.6 Whittington Pain score

Whittington Pain Score

Please enter date of completion: **dd-mmm-yy**

We should be grateful if you would answer the following questions, thinking about how you are at the moment. Please put a circle around your response for each question.

Do you experience pain or discomfort on bladder filling?	**Yes**	**No**
Do you experience pain or discomfort in or over the pubic area?	**Yes**	**No**
Do you experience burning or pain when passing urine?	**Yes**	**No**
Do you experience pain in your back over your kidneys?	**Yes**	**No**
Do you experience pain in the genitals?	**Yes**	**No**
Do you experience pain on the left or right side of the lower abdomen?	**Yes**	**No**
Do you experience general abdominal pain or discomfort?	**Yes**	**No**
Do you experience pain radiating down your legs?	**Yes**	**No**

Score 1 for yes
Score 0 for no

Total score	

9.7 Instructions on clean catch MSU

Midstream Urine Specimen

For correct test results, follow instructions carefully.
A jug, a plastic container and a wet gauze will be given to you

Cleansing before collecting the urine specimen

1. Squeeze and rub some alcohol gel onto your hands once you are in the toilet. There are alcohol gel bottles in each toilet.

2. Thoroughly cleanse the entire genital area using the special gauze that is in the jug.

    - Females – hold the outer edges of labia apart and cleanse from front to back with the gauze. One wipe only!

    - Males – retract foreskin if not circumcised and wipe the end of the penis with the gauze. One wipe only!

Please do not throw the gauze into the toilet bowl. Throw it into the yellow bin which is on the floor of the toilet

Collecting the urine specimen

- Females – continue to hold labia apart while urinating

- Males – continue to retract foreskin while urinating

    1. Urinate (pee) a small amount of urine into the toilet.
    2. Then without stopping, catch some urine into the bowl by passing it into the urine stream.
    3. As the stream comes to the end move the bowl away and urinate (pee) the rest into the toilet
    4. Put the lid on the jug and bring it to a member of staff.

Wash hands after collecting the urine specimen.